Pitx3, its role in lens development and application as a midbrain dopaminergic neuron reporter in embryonic stem cell differentiation

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I declare that the work described in this Thesis is my own except where otherwise stated.

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Table of contents

List of Tables and Figures

Abbreviations

4OHT	4-hydroxy-tamoxifen
6-OHDA	6-hydroxy-dopamine
β-gal	β-galactosidase
BMP	bone morphogenetic protein
AADC	L-aromatic amino acid decarboxylase
ASMD	anterior segment mesenchymal dysgenesis
cdk	cyclin-dependent kinase
CNS	central nervous system
D2R	D2 dopamine receptor
DA	dopaminergic
DAT	dopamine transporter
DAPI	4'-6-Diamidino-2-phenylindole
E	embryonic day
EB	embryoid body
eGFP	enhanced GFP
En	Engrailed
ER	oestrogen receptor
ERK	extracellular-regulated kinases
ES	embryonic stem
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FGF	fibroblast growth factor
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GDNF	glial cell line-derived neurotrophic factor
GFP	green fluorescent protein
GSK-3β	glycogen synthase kinase-3β
hES	human embryonic stem
HygroTK	hygromycin-thymidine kinase
IL-1β	interleukin-1β
IRES	internal ribosomal entry site
IsO	isthmic organiser
IVF	in vitro fertilisation

Klf4	Krüppel-like transcription factor 4
LIF	leukaemia inhibitory factor
MAPK	mitogen-activated protein kinases
mDA	midbrain dopaminergic neurons
MEF	mouse embryonic fibroblast
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NTN	neurturin
ori	the origin of polyoma virus replication
P	postnatal day
pac	puromycin N-acetyltransferase
pCAG	CMV immediate early enhancer and chicken β-actin promoter
pCMV	cytomegalovirus immediate early-enhancer
PD	Parkinson's disease
PGK	phosphoglycerate kinase
polyA	polyadenylation
SDIA	stromal cell-derived inducing activity
Shh	Sonic hedgehog
SN	substantia nigra
TGFβ	transforming growth factor β
TH	tyrosine hydroxylase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area

Abstract

The homeobox gene *Pitx3* has been implicated as a key regulator for lens development because homozygous mutant *aphakia* mice, which are hypomorph for *Pitx3*, fail to develop lenses. One aim of my thesis is to investigate the underlying cellular and molecular mechanism of Pitx3 mediated lens defect by studying knockout mice lacking Pitx3. Chimeric embryos, generated by aggregating the wild type embryos with *Pitx3* heterozygous or *Pitx3* homozygous mutant ES cells, have been used to analyse lens development. *Pitx3* null cells failed to colonise the lens epithelium in *Pitx3* null ↔ wild type chimeric lens, suggesting that Pitx3 is cell-autonomously required for lens epithelial cells. Further study of *Pitx3* null mice revealed an earlier downregulation of the lens epithelial markers PDGFRα and E-cadherin in E11.5 lens epithelium, suggesting the loss of lens epithelial identity in *Pitx3* deficient mice. Furthermore, cell cycle inhibitors $p27^{KIP1}$ and $p57^{KIP2}$ were ectopically expressed throughout the morphologically normal *Pitx3* mutant lens vesicle, suggesting that inactivation of Pitx3 leads to cell cycle exit of epithelial lens cells.

In addition, precocious activation of the fibre cell-specific proteins β- and γ-crystallins was observed in *Pitx3* null lens. β-crystallin expression could be observed as early as E10.5 throughout the entire *Pitx3* null lens vesicle and γ-crystallin was detected in the malformed *Pitx3* deficient lens at E11.5. RNA in situ hybridisation study revealed that the expression of the transcription factor *Foxe3* was lost in *Pitx3* null lens at E10.5, suggesting that Pitx3 maintains the lens epithelial cells partly via the regulation of transcription factor Foxe3 during lens development.

Accordingly, this study provides the cellular and molecular basis for the lens defect observed in *Pitx3* null and *Pitx3* hypomorph *aphakia* mice. Pitx3 is a key transcription factor for the maintenance of lens epithelium and its absence leads to premature activation of fibre cell differentiation programme of lens epithelial cells.

In the other part of my PhD, I have further developed the *Pitx3*-GFP knockin ES cell system with a goal to use this tool for the identification of determinants of midbrain dopaminergic (mDA) neurons, the type of cells lost in Parkinson's disease (PD) patients. Experimental cell therapy and clinical trials have shown that foetal midbrain tissues, but not tissues from other DA neuron containing regions, can functionally restore the lost mDA neurons when transplanted in Parkinson's disease patients. Therefore, it is essential to coax mDA properties on stem cell-derived neurons when considering therapeutic development.

Within the central nervous system, Pitx3 is expressed exclusively in mDA neurons. Using a *Pitx3*-GFP knockin mouse line previously generated in the laboratory I have derived heterozygous and homozygous *Pitx3*-GFP ES cells from mouse blastocysts. In keeping with previous findings in our laboratory, the heterozygous *Pitx3*-GFP ($Pitx3^{GFP/+}$) ES cell-derived GFP positive cells of neuronal morphology can be detected after in vitro differentiation using the PA6 coculture system. Furthermore, I have shown that these cells express tyrosine hydroxylase and midbrain markers Engrailed-1 and Nurr-1, demonstrating their midbrain characteristics. I have also generated supertransfectable $Pitx3^{GFP/+}$ ES cells to offer a rapid and efficient way to express a transgene episomally. The Cre-mediated inducible system of *Pitx3*-GFP reporter ES cells has also been developed in our laboratory and I have shown that

they have high induction efficiency thus allows transgene activation in a temporally controlled manner. The *Pitx3* null ES cells showed impaired potential to differentiate into mDA neurons thus they may be used to evaluate candidate Pitx3 downstream target by gain-of-function test. In summary, I have developed a *Pitx3*-GFP reporter ES cell system to identify mDA regulators functionally by in vitro differentiation.

Chapter 1

Introduction

1.1 Introduction for the transcription factor Pitx3

The homeodomain transcription factor Pitx3 belongs to the family of the newly identified homeobox gene *Pitx*, which has 3 members (Gage et al., 1999). The *Pitx3* gene consists of 4 exons of 121, 130, 203 and 918 bp each (Figure 1-1 A) (Rieger et al., 2001; Semina et al., 1997). The coding region starts within exon 2, while the homeodomain region of the gene is located at exon 3 and exon 4, interrupted by an intron located in the same position as *Pitx1* and *Pitx2*.

Pitx3 was identified by two groups at approximately the same time. Semina et al. screened mouse cDNA libraries with a probe comprising the homeodomain region of *Pitx2* and identified the third member, *Pitx3* (Smidt et al., 1997). Their in situ hybridisation data revealed *Pitx3* expression in the developing lens beginning on E11. Almost at the same time, Smidt et al. screened rat brain cDNA based on brain expressed homeobox genes and isolated this cDNA encoding the *bicoid*-related homeobox gene *Pitx3* (Smidt et al., 1997). They found that Pitx3 has highly restricted brain expression in the midbrain SN and VTA and is superimposed with TH expression, thus Pitx3 could be used as a unique transcription factor marking the mDA neurons at the exclusion of other DA neurons. As well as the developing lens and the midbrain, Pitx3 expression is also reported in the tongue, incisor primordial, mesenchyme around sternum and vertebrae and in the head muscles (Semina et al., 1998).

The Pitx proteins all belong to the *bicoid*-related subclass of homeodomain proteins because they encode the defining lysine at residue 50 within the homeodomain. This residue, at the position 9 within the recognition helix of the homeodomain, is the

major determinant of DNA binding specificity. The Pitx3 protein consists of 302 amino acids, while there are 217 and 317 amino acid isoforms generated by alternative splicing of Pitx2 and Pitx1 is a 315 amino acid protein. These 3 members are highly conserved at the amino acid level. Pitx2 and Pitx3 homeodomain are identical while Pitx1 differs by only 2 amino acids. The C-terminal domains of Pitx1, Pitx2 are around 50% conserved relative to Pitx3; however Pitx1 and Pitx2 isoforms have 70% amino acid identity.

It has been confirmed that *Pitx3* mutation is responsible for the spontaneous mouse mutation *aphakia* phenotype (Rieger et al., 2001; Semina et al., 2000). More recent genomic research on *aphakia* mice has identified two deletion mutations close to and within the mouse *Pitx3* gene; the first one is a 652 bp deletion at 2.5 kb upstream of the transcription start site and the second is a 1423 bp deletion that encompasses the presumed promoter region, the transcription start site, the noncoding exon 1 and a portion of intron 1 (Rieger et al., 2001; Semina et al., 2000)(Figure 1-1 B). This suggests that *Pitx3* mutation is responsible for the *aphakia* phenotype. Therefore, *aphakia* mice are used as a model to investigate *Pitx3* deficiency for mDA development (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003). The functional requirement of Pitx3 in the development of lens and midbrain will be further discussed in section 1.2.4 and 1.3.1, respectively.

It is worth noting that about 5% of wild type levels of *Pitx3* transcripts are still present in *aphakia* mice (Rieger et al., 2001), suggesting that the *aphakia* mutation is a *Pitx3* hypomorph instead of *Pitx3* null. Most studies about *Pitx3* hypomorphic *aphakia* mice are concentrating on the functional roles of Pitx3 in mDA development. The

underlying cellular and molecular mechanism leading to *aphakia* lens defects remains elusive.

In humans, mutation of *PITX3* has been reported with inherited forms of cataracts and ASMD (Addison et al., 2005; Berry et al., 2004; Bidinost et al., 2006; Burdon et al., 2006; Rieger et al., 2001; Semina et al., 1998). This will be further discussed in Chapter 3.

1.2 Introduction for lens

Lens development is an excellent model for developmental biology since the process involves intercellular signalling between different cell layers, invagination in association with morphological changes in cell shape and terminal cell differentiation. It also involves a precisely coordinated development of the anterior neural plate, mesenchyme, and adjacent surface ectoderm (Kondoh, 1999; Lang, 2004; Lovicu and McAvoy, 2005). The fully formed lens consists of post-mitotic differentiated fibre cells that are covered anteriorly by a layer of proliferative immature epithelial cells. Differentiation of epithelial and fibre cells involves acquisition of distinctive morphological and molecular characteristics that are required for the different structures and functions of cells in the two different lens compartments.

1.2.1 Lens development

Lens morphogenesis begins with ectoderm. The optical vesicle, which is an outgrowth from the ventral forebrain that later forms the retina, induces the surface ectoderm to gain the lens competence (Figure 1-2 A). The head surface ectoderm facing the approaching optic vesicle thickens to form the lens placode which subsequently invaginates to forms the lens pit and later the lens cup (Figure 1-2 B).

The lens cup deepens and finally breaks away from the surface ectoderm to form the lens vesicle (Figure 1-2 C). At the same time the optic vesicle invaginates to produce the optic cup with an inner layer, which forms the neural retina and an outer layer, which forms the retinal pigmented epithelium.

Proper lens development requires the mutual inductions between the optic vesicle and the lens placode. The transcription factor Pax6 appears to lie at the top of a regulatory hierarchy (Collinson et al., 2003; Lang, 2004; Ogino and Yasuda, 2000; Reza and Yasuda, 2004a). Mutant mouse studies suggest that FGF (Faber et al., 2001) and BMP7 (Wawersik et al., 1999) receptor signalling are required for lens induction and that they cooperate to promote Pax6 expression (Faber et al., 2001). BMP4 is also required for the optic vesicle to manifest a lens inducing capacity (Furuta and Hogan, 1998).

After lens vesicle formation lens development involves the differentiation of two types of lens cells. A polarity soon develops in the primitive lens. Cells in the posterior segment of the lens vesicle differentiate into elongated, crystallin expressing, primary lens fibres that gradually fill the vesicle, whereas the anterior surface retains a layer of proliferating lens epithelial cells (Figure 1-2 D). Continued elongation of the fibre cells leads to occlusion of the cavity of the lens vesicle (Figure 1-2 E). From this stage onwards, the anterior lens epithelial cells continue to divide throughout life. Most of the proliferation occurs in the epithelial region anterior to the lens equator located between the anterior lens epithelial cells and the posterior lens fibre mass. As the lens epithelial cells migrate posteriorly below the equatorial region, they start to differentiate into secondary lens fibre cells and are

added to the fibre mass throughout life.

When the elongating lens fibre cells reach the midpoint of the lens (Bassnett and Winzenburger, 2003) and meet fibres from the other side, the posterior ends of these cells began to detach from the lens capsule. The continued formation of successive waves of fibre cells causes these elongated cells to be buried deeper within the lens and then degrade all intracellular organelles including the nucleus. The programmed removal of organelles from lens fibre cells is of fundamental importance to the function of the mature lens since it leads to the formation of a transparent region at the centre, which has been termed the organelle-free zone. Disturbances in the programmed removal of organelles during lens development can lead to cataracts. The programmed disappearance of the nucleus in lens fibre cells resembles apoptosis in various ways, including chromatin condensation and DNA fragmentation, which can be visualised using the TUNEL technique, and involvement of the caspases. However, there is a difference between apoptosis, which results in cell death, and the programmed organelle loss, after which lens fibre cells persist throughout the lifetime of the organism. The existence of biochemical similarities to classical apoptosis makes lens fibre cell organelle loss an interesting system for researchers to dissect the molecular events of apoptosis (Wride, 2000). On the other hand, the interpretation of tests about apoptosis, such as caspase expression or TUNEL assay, should be very careful in the context of lens.

The lens and the anterior segment of the eyes are linked in development with a commonality of origin in surface ectoderm components, a necessity for separation of these components for anterior chamber formation and a requirement for signalling

from the lens for anterior segment organisation (Beebe and Coats, 2000). The interaction of the lens vesicle with the overlaying ectoderm results in the induction of the cornea. At the same time, it has been suggested that the anterior segment of the embryonic eye may contain mitogens, which are responsible for maintaining the cell division in the lens epithelium. Some anterior segment disorders arise due to abnormalities in lens development, including mutations in *PAX6* (Hanson et al., 1994; Ton et al., 1991), *MAF* (Jamieson et al., 2002; Lyon et al., 2003), *PITX3* (Semina et al., 1998), and *FOXE3* (Semina et al., 2001).

1.2.2 Transcription factors involved in lens development

- Pax6

Pax6 is a member of the Pax family, the paired-type homeobox-containing transcription factors. In *Drosophila*, there are two *Pax6* homologues called *eyeless* and *twin-of-eyeless*. Gain-of-function experiments have shown that *Pax6* is sufficient for eye formation. Misexpression of Pax6 in *Xenopus* embryos leads to the formation of ectopic eyes with fully differentiated lenses and retina tissues (Chow et al., 1999).

In mice, *Pax6* is expressed broadly in the head ectoderm from E8.0 which becomes progressively restricted to the developing lens placode and nasal placode at E10. It is also expressed later in the optic vesicle, but its expression in surface ectoderm is earlier than in the optic vesicle apposition (Li et al., 1994). Heterozygous *Pax6* mutation results in the phenotype of *Small eye (Sey)* (Hill et al., 1991). Mouse embryos homozygous for the *Sey* mutations do not develop lens and nasal placodes, resulting in a lack of eyes and nose, and die perinatally due to an inability to breathe (Hill et al., 1991; Hogan et al., 1986). In these *Sey*/*Sey* mice, there is no lens

induction and no sign of thickening of the ectoderm to form lens placodes in spite of the apposition of optic vesicles, suggesting that Pax6 activity in the presumptive lens ectoderm but not in the optic vesicle is required for the initiation of lens induction.

Assessment of *Pax6* mRNA expression patterns in wild type and homozygous *Pax6*$^{Sey\text{-}1Neu/Sey\text{-}1Neu}$ mutant mice shows that *Pax6* expression in the surface ectoderm can be divided into at least two stages (Grindley et al., 1995; Grindley et al., 1997; Gumbiner, 2000). The first stage corresponds to *Pax6* in the surface ectoderm, before close contact with the optic vesicle occurs (defined as *Pax6*$^{\text{pre-placode}}$). The second stage occurs after contact, and correlates with the formation of the lens placode (defined as *Pax6*$^{\text{placode}}$). Functional *Pax6* in the first stage is required for continued placodal *Pax6* expression and subsequent lens development.

Tissue recombination studies in rats indicate that the ectoderm from homozygous (*rSey*/*rSey*) never differentiates into lens tissue even if it is cultured with normal optic vesicle tissue from wild type embryos, whereas lens differentiation occurs when wild type or heterozygous ectoderm is cultured with homozygous *Pax6* mutant optic vesicles (Fujiwara et al., 1994). It suggests that *Pax6* is required by the head ectoderm for responding to the inductive effect of the optic cup, thus Pax6 expression confers lens competence to the head ectoderm. Aggregation of wild type and *Sey*/*Sey* embryos results in chimeric mice in which *Sey*/*Sey* mutant cells are excluded from the lens placode (Collinson et al., 2000) and the maturing lens at E12.5 (Quinn et al., 1996), respectively. The chimera studies also revealed that Pax6 is required for the maintenance of contact between the lens placode and the optic vesicle (Collinson et al., 2000). Deletion of *Pax6* in the prospective lens ectoderm by Cre-loxP

tissue-specific gene targeting results in the absence of the lens placode formation, which supports the cell-autonomous requirement for Pax6 in placode initiation (Ashery-Padan et al., 2000).

An ectoderm enhancer (EE) of *Pax6* has been deleted by gene targeting (Dimanlig et al., 2001). The homozygous mutant mice ($Pax6^{\Delta EE/\Delta EE}$) have a reduction in lens placode thickness and proliferation rate, a smaller lens, delayed primary fibre cell differentiation, a persistent connection between the lens and surface ectoderm, and loss of expression of *Foxe3*, which is another transcription factor required for lens development.

At later stages, Pax6 continues to be expressed in the entire lens vesicle and, when the lens matures, Pax6 expression is confined to the lens epithelium (Grindley et al., 1995). *βB1-crystallin* promoter analysis shows that Pax6 represses the expression of the lens fibre cell-specific marker β-crystallin, which is first expressed after *Pax6* expression has ceased (Cui et al., 2004; Duncan et al., 1998). Taking these data together, it has been suggested that the function of Pax6 at later stages is to maintain the lens epithelium state by inhibiting fibre cell-specific genes, and that the downregulation of Pax6 in differentiated lens fibre cells during development allows β-crystallin activation by other positive regulators such as Prox1 and c-Maf.

Since *Pax6* homozygous null embryos are completely lens-less, the *Pax6* heterozygous lens, which shows haploinsufficient phenotype, serves as another model system to study the functional role of Pax6 and to identify genes regulated by Pax6. $Pax6^{+/-}$ chimera data demonstrates a cell autonomous deficiency in lens placode formation as well as in lens epithelium after closure of the lens pit

(Collinson et al., 2001). Reduced levels of expression of *Pitx3* in *Pax6* heterozygous lens by microarray and RT-PCR analysis have also been shown (Chauhan et al., 2002a; Chauhan et al., 2002b). Furthermore, two conserved Pax6 binding sites are found in the 5'-flanking sequences of the human and mouse *PITX3/Pitx3* genes. These data suggest that Pax6 may directly regulate Pitx3.

In humans, one case of a stillborn foetus of homozygous *Aniridia*, which is associated with the *PAX6* mutation, has been reported with severe craniofacial abnormalities, including a total absence of the eyes and nose. The phenotype is similar to homozygous *Pax6* mutation in mice. Heterozygous loss of function of *PAX6* is responsible for anterior segment disorder of eyes, including ASMD and Peters' anomaly (Hanson et al., 1994; Ton et al., 1991).

- Sox1 and Sox2

Sox1, Sox2 and Sox3 belong to the B group of Sox proteins which have previously been described as δEF2 that can activate δ-crystallin enhancer and γ-crystallin promoter (Kamachi et al., 1998). In mice, Sox2 expression starts in Pax6 expressing head ectoderm just before the lens placode is formed and its activation defines the lens fate of the ectoderm. Subsequently Sox2 is highly expressed in the lens pit and lens vesicle. Later on, Sox2 expression is downregulated and taken over by Sox1, which is the major Sox protein expressed throughout the lens, and the expression is significantly higher in the fibre compartment. Sox3 is also expressed in the vesicle-facing ectoderm in chick embryos, but its expression is not detectable in mouse embryos. Optic vesicle ablation experiments in the chick have shown that induction of Sox2/3 activation depends on the effect of the apposing optic vesicle.

Sox2 homozygous null embryos die soon after implantation, reflecting the unique expression of Sox2 in the inner cell mass and epiblasts (Avilion et al., 2003). Recently, *Sox2* hypomorphic and conditional knockout mice have been generated. These mice showed a reduced eye size caused by impaired proliferation of retinal neural progenitors, but no lens phenotype was observed (Taranova et al., 2006). *Sox1* knockout mice are viable and their major phenotype is the defect in lens fibre development (Nishiguchi et al., 1998). Lens fibre formation is initiated normally upon withdrawal from the cell cycle, but is prematurely arrested before cell elongation, thus the lens cavity remains empty and gives rise to a hollow lens. All *γ-crystallin* genes are severely affected but the expression of *α-* and *β-crystallin* occurs normally. This suggests that Sox1 is required for γ-crystallin expression and/or differentiation of lens fibre cells.

- Six3

Six3 was originally cloned as the vertebrate homologue of *Drosophila's Sine oculis* (*so*) homeobox-containing gene (Oliver et al., 1995). In mice, Six3 is progressively expressed in the optic stalk, lens pit, optic vesicle and is later restricted to the neuroretina and lens epithelium. Misexpression of mouse Six3 in medaka fish can direct ectopic lens formation in a cell non-autonomous manner, suggesting that it may induce a soluble factor that changes the bias of the optic placode toward a lens type fate (Oliver et al., 1996). Six3 can significantly repress the promoter activity of the γF-crystallin gene *crygf* in a promoter assay (Lengler et al., 2001) and suppress lens fibre differentiation when overexpressed in the chick lens primordium (Zhu et al., 2002). Together with the fact that Six3 and γ-crystallin expression is inversely related during lens development, it has been suggested that Six3 may prevent

γ-crystallin expression in the lens epithelium whilst its downregulation in differentiated lens fibre cells allows γ-crystallin genes to be activated by other positive regulators such as Prox1 and c-Maf. Thus the primary function of Six3 may be to maintain the proliferative state of lens epithelium (Reza and Yasuda, 2004a).

Six3 is also essential for the establishment and maintenance of the anterior neuroectodermal identity including the presumptive forebrain and developing retina. Six3 expression can be detected as early as E7.0 in the prospective anterior neuroectodermal region including the area which later on gives rise to the optic vesicle and retina primordium. The set of regulatory molecules that control this expression may be different from the set that regulates the expression of head ectoderm that includes the area which later on and after thickening gives rise to the lens placode (Lagutin et al., 2001). Overexpression of *Six3* results in expanded and ectopic retinal primordial in medaka fish (Loosli et al., 1998; Loosli et al., 1999) and *Xenopus* (Bernier et al., 2001), and promotes the formation of ectopic optic vesicle-like structures in the midbrain-hindbrain region of developing mouse embryos (Lagutin et al., 2001). Inactivation of *Six3* leads to the complete absence of the forebrain and eyes (Carl et al., 2002). It has been reported that Six3 regulates the proliferation of retina precursor cells by antagonising the cell cycle inhibitor geminin (Del Bene et al., 2004).

In humans, *SIX3* expression has been detected in the retina (Granadino et al., 1999). Mutation of human *SIX3* is associated with human holoprosencephaly type 2 (*HPE2*), of which the manifestation includes severe malformation of the brain, microphthalmia and craniofacial defects. This suggests that SIX3 is essential for the

development of anterior neural plate (Granadino et al., 1999; Wallis et al., 1999).

- Prox1

Prox1 is a homeodomain protein related to *Drosophila prospero*. During mouse development, Prox1 is expressed in the lens placode and lens vesicle but the expression level is highest in the lens equator in differentiating lens (Duncan et al., 2002; Wigle et al., 1999). Indeed, loss-of-function study of *Prox1* in mice has demonstrated an involvement of Prox1 in the transition of the proliferative lens epithelium to the post-mitotic lens fibre cells.

Histological analysis of *Prox1* homozygous mutant eyes has revealed that the initial induction of the lens vesicle is unaffected. However, the mutant lens cells failed to polarise and elongate properly, resulting in a hollow lens. *Prox1* inactivation causes downregulated expression of the cell cycle inhibitors $p27^{KIP1}$ (also known as cdkn1b) and $p57^{KIP2}$ (also known as cdkn1c), suggesting that Prox1 activity is necessary for cell cycle withdrawal of the lens epithelial cells. At E14.5 the *Prox1* mutant lens cells uniformly express the epithelial marker E-cadherin. RT-PCR analysis of the mutant lens shows slightly downregulated but detectable expression of the genes encoding *α*-, *β*- and most of the *γ- crystallins*. However, expression of the genes encoding γB- and γD- crystallin are not detected in mutant lens. The expression of most genes encoding *crystallins* suggests that fibre cell differentiation is initiated but then arrested in *Prox1* null lens despite of the loss of cell cycle inhibitors $p27^{KIP1}$ and $p57^{KIP2}$.

- Foxe3

The forkhead gene *Foxe3* starts to be expressed in the prospective lens surface ectoderm, where later, the lens placode is formed (Blixt et al., 2000; Brownell et al., 2000). Subsequently, Foxe3 expression is confined to the lens vesicle as it is detached from the surface ectoderm. Initially it is evenly distributed throughout the lens vesicle, and then the expression is switched off in the posterior cells of the lens vesicle as these cells start to differentiate into lens fibres, but remains high in lens epithelial cells. By E14.5 the Foxe3 expression is restricted to anterior lens epithelium, where it persists into adulthood.

Foxe3 is mutated in the spontaneous mouse mutation *dysgenetic lens* (*dyl*) (Brownell et al., 2000). The phenotype of *dyl* is manifested as a persistent connection between the lens and the corneal epithelium, which results from failure of the lens vesicle to close and separate from the ectoderm. The lens structure is irregular in shape and reduced in size. It has been suggested that the primary defect of *dyl* mouse lens morphology is due to failure of the lens epithelium to proliferate, which subsequently leads to a reduction in the number of secondary lens fibres.

In the *dyl* mutants the expression of cell cycle exit marker $p27^{KIP1}$ extends anteriorly into the epithelium, the *pdgfra* expression is diminished compared to the wild type, and the epithelial cells express a higher level of the *crya1* gene, which encodes *α1-crystallin* (Blixt et al., 2000). There is also inappropriate upregulation of β-crystallin in the lens epithelial cells (Brownell et al., 2000). This suggests that the functional role of Foxe3 may be to maintain the border separating the dividing cells of the epithelium from the posterior differentiating cells, by preventing the expression in epithelial cells of genes involved in growth inhibition and

differentiation (Blixt et al., 2000). The expanded expression pattern of *Prox1* in the lens epithelium of *dyl* mice indicates that Foxe3 may be a suppressor of *Prox1* in this location. *Foxe3* expression is absent in *Pax6* mutant embryos (Brownell et al., 2000; Dimanlig et al., 2001), showing that *Foxe3* is located downstream of the placodal phase of *Pax6* expression in a genetic pathway regulating lens development. *Foxe3* knockout mice have recently been generated by targeted deletion (Medina-Martinez et al., 2005). The morphological and molecular changes observed in the lenses of *Foxe3* null embryos are similar to those observed in the *dyl* mouse strain.

In a recent zebrafish experiment, *foxe3* knockdown resulted in a smaller lens with increased numbers of nucleated cells and disordered fibre cell arrangements. The most interesting finding is that the knockdown pitx3 protein in zebrafish results in the elimination of *foxe3* expression, suggesting that *foxe3* lies genetically downstream of *pitx3* in a zebrafish lens development pathway (Shi et al., 2006a).

In humans, *FOXE3* is expressed in the lens epithelium (Semina et al., 2001). Mutations in *FOXE3* have been identified in several families with ASMD and cataracts characteristic of Peters' anomaly, similar to those of the individuals with *PITX3* mutation (Ormestad et al., 2002; Semina et al., 2001).

- Maf

The *Maf* oncogenic family originally emerged with the isolation of the *v-maf* gene from the genome of acute transforming retrovirus AS42, which causes avian musculoaponeurotic fibrosarcoma (Nishizawa et al., 1989). All the proteins in this family share a highly conserved basic region of a leucine zipper domain (bZIP) at their C-terminals, which allows them to bind to the target DNA, the Maf-responsive

element (MARE), either as homodimers or as heterodimers with other transcription factors. Maf family transcription factors are subdivided into two major groups based on their molecular size: small Mafs or large Mafs. Large Mafs identified in human and mouse include c-Maf, MafB, MafA and NRL (neural retina leucine zipper), whereas c-Maf, MafB and L-Maf have been identified in chicks and *Xenopus* (Reza and Yasuda, 2004b). *Maf* genes are expressed in a highly regulated temporospatial manner, suggesting different Mafs have distinct roles in lens development.

L-Maf is the first chick Maf protein to be expressed in developing lens cells. When the invaginating optic vesicle approaches, L-Maf is expressed in the overlying head surface ectoderm, regulated by Pax6 (Reza et al., 2002). Ectopic expression of L-Maf in different parts of the head ectoderm induces *Prox1* and *crystallin* genes expression (Reza et al., 2002). After the lens vesicle stage, L-Maf essentially regulates the expression of crystallin and fibre-specific genes.

Mouse c-Maf and chick L-Maf have similar functions. In mice, c-Maf expression is first evident in the presumptive lens ectoderm before its invagination. At E11.5 the expression is preferably higher in the fibre cells and less in epithelial cells (Kawauchi et al., 1999). However, mouse c-Maf does not play as an important role in lens placode formation as chick L-Maf does because lens development is not impaired until the lens vesicle stage in *c-Maf* mutant mice. c-Maf function is crucial for lens fibre differentiation and crystallin expression in mice. In the *c-Maf* knockout mice, the initial stages of primary fibre cell function are normal but fibre cell differentiation and elongation cease by E12.5, giving rise to the persistence of a hollow lens structure. This phenotype partially overlaps with those reported for gene

targeting of *Prox1* and *Sox1*. However, RNA in situ hybridisation at E13.5 shows no difference between the *c-Maf* homozygous mutant and the heterozygous controls for the expression of *Prox1* or *Sox1* (Ring et al., 2000). In *c-Maf* mutant mice, weak expression of α- and β-crystallin has been observed but γ-crystallin is almost absent, demonstrating that γ-crystallin gene transcription is under strict c-Maf regulatory control (Kawauchi et al., 1999; Ring et al., 2000).

Mouse MafB mRNA is apparent in the lens epithelial cells at E10.5 but not in the lens fibre cells, while NRL is absent in early lens lineage during development (Kawauchi et al., 1999). Since MafB expression in mice is exclusively detected in the lens epithelium, it has been suggested that it is involved in the maintenance of lens epithelial cells (Reza and Yasuda, 2004b) but further investigation is required.

In humans, inherited cataract of the lens along with other ocular abnormalities has been identified due to *MAF* mutation (Jamieson et al., 2002; Lyon et al., 2003).

1.2.3 Signal pathways for lens development

Lens polarity and growth pattern are maintained throughout life by growth factors that are present in the ocular environment. It is well known that growth factors modulate each other's bioactivity and bioavailability. The complex process of fibre differentiation depends on the synchronised and integrated action of a number of distinct growth factor-induced pathways.

- BMP pathway

Members of the TGFβ superfamily appear to have a prominent role in regulating aspects of lens induction and differentiation. BMP proteins form soluble dimers

which are involved in autocrine and paracrine signalling during development. Binding of BMPs induces the formation of complexes of type I and type II receptors. Activated type II receptors transphosphorylate type I receptors, which propagate the signal to the nucleus via a family of structurally related proteins known as SMADs.

BMPs, TGFβs and their respective receptors are expressed in the lens (de Iongh et al., 2001; Faber et al., 2002). BMP4 and BMP7 are crucial for the early steps of the lens induction process. In mice, BMP4 is highly expressed in the dorsal region of the optic vesicle, but weakly expressed in the overlying surface ectoderm, whereas BMP7 is expressed in the head ectoderm where later on, the lens placode forms (Furuta and Hogan, 1998; Wawersik et al., 1999). In *BMP7* mutant mice, neither Pax6 expression nor lens placode formation is observed, suggesting that BMP7 functions upstream of Pax6 expression in the presumptive lens ectoderm (Wawersik et al., 1999). On the other hand, *BMP4* null mutant embryos show no lens placode induction associated with the lack of Sox2 expression in the head ectoderm. Lens formation from presumptive lens ectoderm of *BMP4* null mutants can be rescued by recombining them with wild type optic vesicle in explant culture. Explantation of eye rudiments from *BMP4* null mice in the presence of exogenous BMP4 supply rescues the Sox2 expression, suggesting a functional role of BMP4 for Sox2 induction and lens development (Furuta and Hogan, 1998). In contrast, applying BMP4 beads without optic vesicles does not induce lens formation. These observations indicate that BMP4 and some unknown signals from the optic vesicle are required to form lens placode from the ectoderm.

In addition to their important roles in lens induction, BMPs are also important for lens fibre cell elongation and differentiation (Faber et al., 2002). Phosphorylated SMAD1 (pSMAD1), a target of BMP signalling, localises to the nucleus of elongating lens fibre cells (Belecky-Adams et al., 2002). In mice, in vitro experiments have shown that primary lens fibre cell elongation can be suppressed in the presence of Noggin, which is a BMP ligand inhibitor (Faber et al., 2002). Primary fibre cell elongation is also inhibited when a dominant-negative form of BMP receptor type I (BMPR1B, Alk6) is overexpressed in the lens (Faber et al., 2002). Targeted deletion of *Alk3* (BMPR1B), the most abundant type 1 BMP receptor, by the Pax6 P0 promoter, results in a smaller lens with thin epithelial layers (Beebe et al., 2004). *Alk3* null lens fibre cells become vacuolated and begin to degenerate by the first postnatal week. These studies show that BMP signalling also contributes to the later development of lens epithelial and fibre cells.

Other members of TGFβ superfamily are also important for lens fibre differentiation. Follistatin, a binding protein that binds to the members of the TGFβ superfamily, cooperates with Noggin to block the elongation of cultured chick lens epithelial cells (Beebe et al., 2004). Overexpression of dominant-negative type II and type I TGFβ receptors shows disrupted fibre cell differentiation including attenuated fibre cell elongation (de Iongh et al., 2001). However, targeted deletion of the type II TGFβ receptor does not result in abnormalities in lens growth and differentiation (Beebe et al., 2004). Careful evaluation by further experiments is needed to have a clearer picture about the regulation of these signalling pathways.

- Wnt pathway

Wnts function as ligands for members of the Frizzled (Fz) family of seven-transmembrane receptors. The presence of Fz receptors and LDL (low density lipoprotein)-related protein co-receptors (LRPs), 5 or 6 are required to activate the Wnt/β-catenin pathway (Cong et al., 2004; Tamai et al., 2000), which is commonly referred to as the Wnt canonical pathway. In situ hybridisation shows that Wnt5a, Wnt5b, Wnt7a, Wnt7b, Wnt8a and Wnt8b gene, and frizzled receptors Fz1-Fz8, are expressed throughout the embryonic lens up to E12.5 but then become predominantly restricted to the lens epithelium and the equatorial region (Chen et al., 2004). The negative regulators of Wnt signalling, Dickkopfs (Dkks) 1, 2 and 3, are strongly expressed in the lens epithelial cells and elongating primary lens fibres but downregulated in mature fibre cells (Ang et al., 2004). It has been shown that LRP6 is expressed, and active β-catenin is present in the lens epithelium during development (Stump et al., 2003). Mice with a null mutation of *LRP6*, which is a Wnt co-receptor, have small eyes and an aberrant lens characterised by an incompletely formed anterior lens epithelium. Therefore it has been suggested that the Wnt/β-catenin pathway may have a role in maintenance of anterior epithelial cells or in preventing aspects of fibre differentiation. Further investigation is needed to confirm the functional requirement of the Wnt signalling pathway in the lens epithelium.

- FGF pathway

Members of the FGF family play key roles in mammalian lens biology. The picture that is emerging so far is that FGF appears to be a key initiator for lens development. The expression of Pax6 and Sox2 in the lens placode is significantly decreased when the FGF receptor is inactivated in the presumptive lens ectoderm, suggesting that

Pax6 and Sox2 lie downstream of the FGF signalling in the lens induction pathway (Faber et al., 2001).

At later stages, FGF1, FGF2 and high affinity FGF receptors are shown to be expressed throughout the eyes, in particular in the lens (de Iongh et al., 1997; de Iongh et al., 1996). In a rat lens epithelial explant culture system, FGF is shown to induce proliferation or differentiation responses in lens epithelial cells in a concentration-dependent manner. A low concentration of FGF induces lens epithelial cell proliferation, whereas subsequent higher doses induce lens epithelial cell migration and fibre cell differentiation, which includes morphological change accompanied by the accumulation of fibre cell-specific β- and γ-crystallins (McAvoy and Chamberlain, 1989). Since more FGF can be recovered from the vitreous than aqueous humour, these dose-response characteristics of lens cells to FGF lead to the development of the FGF gradient hypothesis, which proposes that the anteroposterior patterns of lens cell behaviour are determined by an anteroposterior gradient of FGF stimulation (McAvoy et al., 2000). It is possible that low concentrations of FGF in the anterior chamber of the eye maintains the proliferation of lens epithelial cells, and in the equatorial region cells are exposed to higher levels of FGF which leads to the definite process of terminal differentiation.

Overexpression of a truncated FGF receptor that acts in a dominant-negative manner (Stolen and Griep, 2000) and overexpression of a specific secreted FGF receptor SFGFR3 (Govindarajan and Overbeek, 2001) lead to inhibition of fibre cell differentiation in vivo. These experiments indicate that FGF ligand and the FGF receptor signalling is essential for lens fibre cell differentiation. However, many

FGFs have overlapping effects in the lens (Lovicu and Overbeek, 1998) and mice null for a number of different FGFs have no abnormal lens phenotype (Zhao et al., 2002). Additionally, no single *fgfr* gene is essential for lens fibre cell differentiation. However, the triple knockout of *Fgfr1*, *Fgfr2* and *Fgfr3* shows no fibre differentiation and confirms that different FGFRs play an essential but redundant role in fibre differentiation (Zhao et al., 2003). To date it is still poorly understood which of the currently known FGF family members are involved in the regulation of lens fibre cell differentiation in vivo.

The intrinsic signalling pathway associated with FGF receptor induces mitogen-activated protein kinases (MAPK), of which the sequential phosphorylation of its components are then translocated from the cytoplasm to the cell nucleus where it activates nuclear transcription factors leading to changes in gene expression. To date several distinct mammalian MAPKs have been characterised and among them, the extracellular-regulated kinases (ERKs), are the most abundant MAPK in the lens. Rat immunohistochemistry experiment reveals that phosphorylated ERK (pERK) is observed throughout the lens epithelial cells including the equatorial region, and downregulated in differentiated lens fibre cells (Lovicu and McAvoy, 2001). In rat lens explant experiments, high differentiation-inducing doses of FGF induce a greater level of ERK phosphorylation than a lower proliferation-inducing dose (Lovicu and McAvoy, 2001). Additionally, FGF-induced lens fibre cell differentiation, including elongation and expression of the fibre cell-specific marker filensin, is also dependent on ERK activation. However, FGF-induced β-crystallin accumulation in rats is independent of the ERK pathway (Lovicu and McAvoy, 2001), in keeping with the chick experiment which shows that FGF-induced δ-crystallin

expression does not require ERK activation (Le and Musil, 2001). Thus FGF-induced lens fibre cell differentiation may involve other yet to be identified signalling pathways regulating the expression of the fibre cell-specific crystallin. These studies demonstrate the uncoupling of the fibre cell differentiation process in the lens and highlight that multiple signalling pathways are involved in the regulations.

1.2.4 The *Pitx3* hypomorphic *aphakia* mice

The spontaneous mouse mutation *aphakia* (*ak*) was first observed in a colony of strain 129/Sv-S1^{j} mice in 1968 (Varnum and Stevens, 1968). Because of difficulties in maintaining the *aphakia* line, mutants were outcrossed to C57BL/Ks animals. The *aphakia* mice appear to be small, emaciated, and have reduced fertility. They are characterised by small sized eyes with lack of lens and anterior segment structures. Morphologically *aphakia* and normal lens are grossly indistinguishable until E10, the lens vesicle stage. However, a significantly greater number of malorientated mitoses is found in the lens placode and the early lens cup in homozygous *aphakia* mice (*ak/ak*) (Zwaan and Kirkland, 1975). By E11, the lumen of the lens vesicle is filled with disorganised groups of rounded cells. Additionally, the extracellular matrix material that forms a firm attachment between the lens vesicle and optic vesicle during invagination of the lens vesicle is abnormal (Zwaan and Webster, 1984). The cells of the posterior wall never elongate to become lens fibres. Abnormal lens morphogenesis in the *aphakia* mutant results in a club-shaped elongated lens remnant that remains attached to the face surface epithelium by a persistent connecting stalk. Subsequently the disorganised rudimentary lens disappears during later development. The growth of the retina continues and the retina tissue eventually fills the space of the whole eye ball without formation of the vitreous body. The folding of the retina may be caused by the

absence of the lens and continued growth of the retinal layer, or by an alternation of a lens-derived signal.

Since separation of the lens vesicle from the surface ectoderm is essential for formation of anterior segment, the anterior eye structures, including the cornea, iris, and pupillary opening, never differentiate properly in *aphakia* mice (Zwaan and Webster, 1984; Zwaan and Webster, 1985). It has been suggested that the persistent lens stalk, which does not detach from the surface ectoderm, is caused by the differences in the intercellular deposition and composition of the extracellular matrix between the *aphakia* and normal mice at the lens placode stage (Zwaan and Webster, 1984). Furthermore, it has been reported that *aphakia* mutant lens remnants do not express crystallin protein, suggesting impairment in the function of lens differentiation (Zwaan, 1975). Previous work on chimeras showed that homozygous *aphakia* (*ak*/*ak*) cells did not contribute to the lens, indicating that the gene acts cell autonomously in the lens (Liegeois et al., 1996).

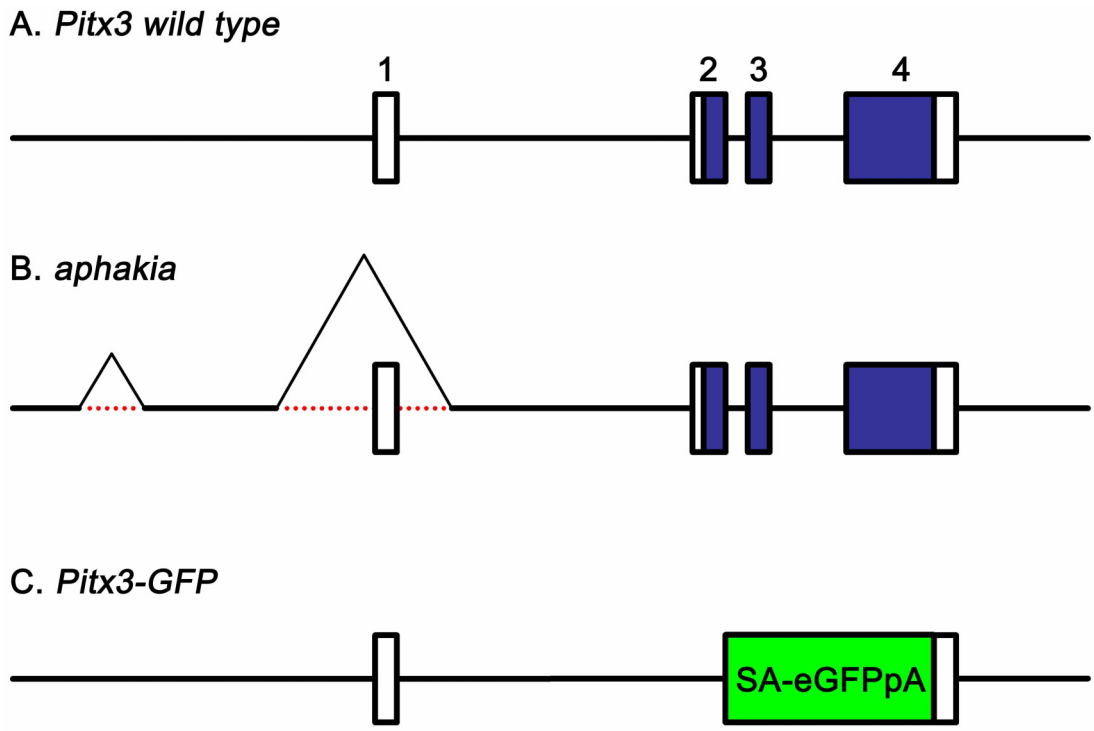

Figure 1-1 The genomic structure of the *Pitx3* wild type, *aphakia* and the *Pitx3*-GFP targeted locus

(A) The four boxes represent *Pitx3* exons. The filled blue regions indicate coding sequence, whilst the blank regions represent non-coding sequence. (B) The two deletions previously identified in aphakia are marked by triangles (drawing not to scale). (C) In the *Pitx3*-GFP targeted allele, the entire coding sequence of *Pitx3* is replaced with *eGFP* cDNA, marked by filled green box.

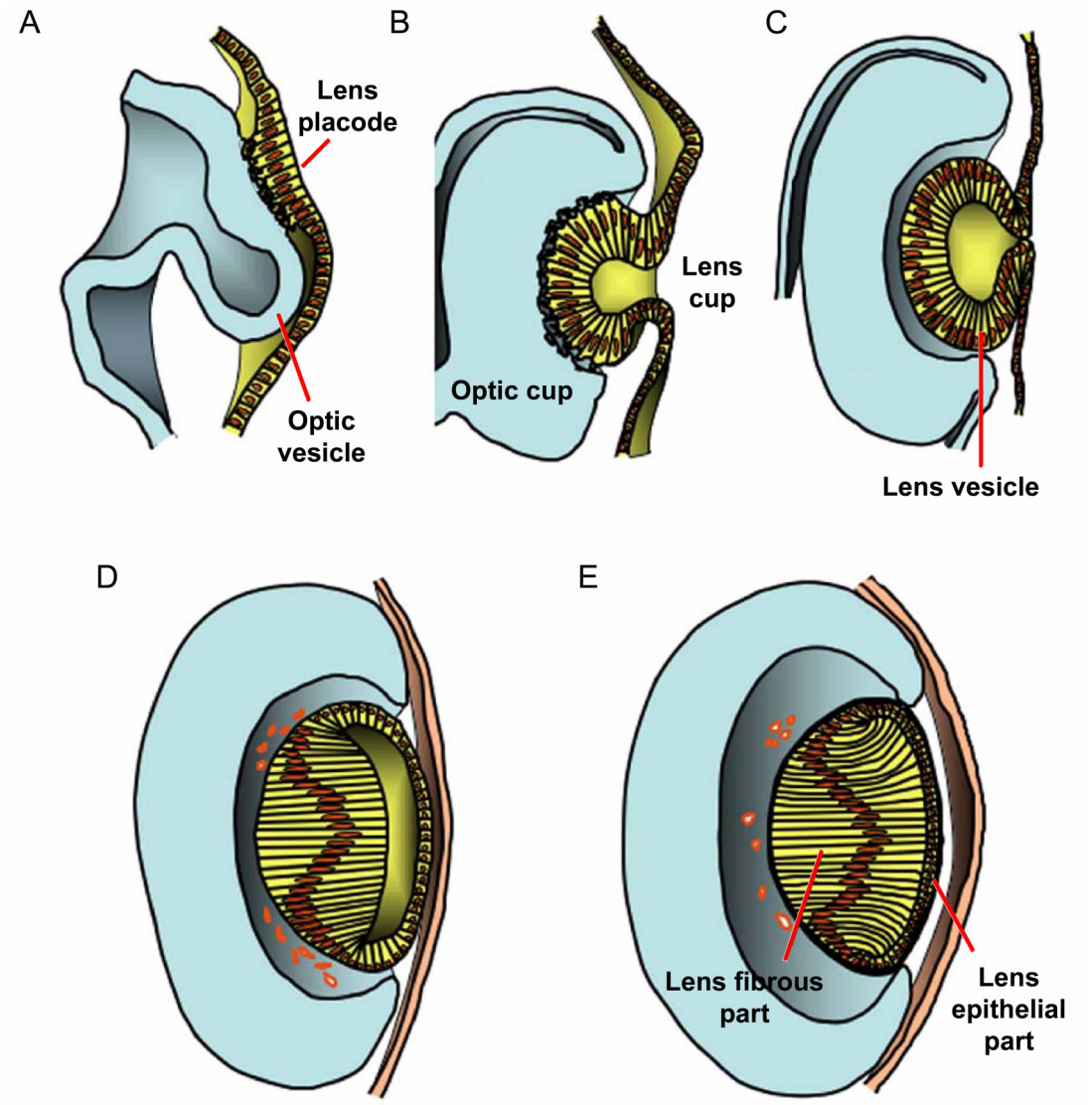

Figure 1-2 Schematic diagrams of lens development

The head surface ectoderm facing the approaching optic vesicle, which grows out from the developing forebrain, thickens to form the lens placode (A). Invagination of the placode with optic vesicle leads to the formation of the lens cup and the optic cup, respectively (B). The lens cup deepens and finally breaks away from the surface ectoderm to form the lens vesicle (C). Cells in the posterior part of the lens vesicle elongate to form primary lens fibre cells (D). Continued elongation of the fibre cells leads to occlusion of the cavity of the lens vesicle (E).

Adapted from (Lovicu and McAvoy, 2005)

1.3 Introduction for embryonic stem cells and midbrain dopaminergic neuron differentiation

With the mouse and human genome project well underway, the goal of understanding gene function in mammalian development appears a real possibility. It has revolutionised our ability to probe the role of a particular gene in development by current technological advances, among which the derivation of the embryonic stem (ES) cells from inner cell mass of blastocysts has opened up the possibilities for introducing designed genes into the mouse germ-line to produce transgenic animals. In addition to their use in gene targeting, ES cells can give rise to a variety of somatic cell types in culture. The combination of genetic manipulation of ES cells and their in vitro differentiation into somatic cell types lends a powerful system to identify key cell fate determinants and to model embryonic development.

1.3.1 Development of mesencephalic dopaminergic neuron

Dopaminergic (DA) neurons play crucial roles in motor and behaviour processes (Morris et al., 2006). They are usually identified by the presence of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine biosynthesis. They were originally grouped and numbered as if they were parts of the noradrenergic system because identification was based on histofluorescence, which does not distinguish dopamine from norepinephrine very well. In the dorsal hypothalamus, the A11 and A13 DA neurons send descending axons to autonomic areas of the lower brain stem and the spinal cord (Figure 1-3 A), regulating sympathetic preganglionic neurons. Along the wall of the third ventricle, the A12 and A14 DA neurons are components of the tuberoinfundibular hypothalamic neuroendocrine system. Some of them release dopamine as a prolactin releasing factor in the hypophyseal portal circulation.

There are also DA neurons in olfactory tubercle (A15), olfactory bulb (A16) and retina (A17) (Prakash and Wurst, 2006a).

The largest group of DA neurons in CNS are the mesencephalic DA neurons in the ventral midbrain, located in the retrorubral field (A8), the substantia nigra (SN; A9), and ventral tegmental area (VTA; A10)(Figure 1-3 B). The VTA DA neurons project to limbic structures such as nucleus accumbens and olfactory tubercle, and are involved in emotional behaviour and mechanisms of reward. Dysfunction of this mesolimbocortical system has been implicated in drug addition, depression and psychotic disorders such as schizophrenia. The SN DA neurons project into the dorsolateral striatum, forming the nigrostriatal pathway mediating the control of voluntary movement (Simon et al., 2003; Wallen and Perlmann, 2003). Impairment of nigrostriatal pathway results in Parkinson's disease (PD).

The correct specification and development of mesencephalic dopaminergic (mDA) neurons depends upon the proper development of the ventral midbrain which involves a number of signalling/transcription factors. mDA neurons are generated at the ventral mesencephalon just rostral to the isthmic organiser (IsO), which is a signalling centre located at the border between the midbrain and the hindbrain and its position determines the location and size of the mDA neuron population (Brodski et al., 2003). At the end of gastrulation, embryonic day (E)7.5 in mouse, the future position of the IsO is identified by the opposing expression domains of two transcriptional repressors: Otx2 in the presumptive fore- and midbrain, and Gbx2 in the presumptive hindbrain and spinal cord (Li and Joyner, 2001). At E8, the transcription factor Pax2 is expressed across the Otx2/Gbx2 border and expression of

the secreted molecule Wnt1 is predominantly limited to the Otx2 domain (Wurst and Bally-Cuif, 2001). Following this, the transcription factors En1, En2 and Pax5 are expressed across the Otx2/Gbx2 border, and fibroblast growth factor (FGF8) is expressed on the Gbx2 positive side (Wurst and Bally-Cuif, 2001). By E9.5 the expression of Wnt1 and FGF8 domains have sharpened into rings on the rostral and caudal sides of the IsO, respectively (Wurst and Bally-Cuif, 2001). The intersection of the floor plate-derived signal Sonic hedgehog (Shh) with the isthmus-derived FGF8 creates an inductive centre for mDA neurons just rostral to the IsO. It has been shown that Shh is necessary and sufficient for the induction of mDA neurons along the dorso-ventral axis, whereas FGF8 is responsible for the position of mDA neurons along the anterior-posterior axis of the neural tube (Ye et al., 1998). However, recent studies have highlighted that the mDA-inducing effects of Shh and FGF8 are complex and that interactions with other factors are required for correct cell-type specification in the midbrain.

The secreted glycoprotein Wnt1 is expressed around the IsO, predominantly on the midbrain side, and later refined to a narrow ring on the rostral site of the IsO. A key role for Wnt1 in midbrain development has been demonstrated by loss-of-function studies which reveal a failure by *Wnt1* deficient embryos to develop any midbrain or anterior hindbrain structure (McMahon and Bradley, 1990; McMahon et al., 1992). In the *Wnt1* null mice, few TH expressing cells are still generated, but they fail to initiate expression of Pitx3 (Prakash et al., 2006). In addition to Wnt1, Wnt5a is also expressed at a high level in the ventral midbrain, with a peak at E11.5 in rat (Castelo-Branco et al., 2003). Addition of Wnt5a to rat E14.5 ventral midbrain primary cultures has resulted in an increase in the number of TH expressing neurons.

Furthermore, there was an increase in the overall expression levels of *Pitx3* in cultures treated with exogenous Wnt5a. From these studies the authors suggested that Wnt5a increases the total number of mDA neurons by promoting the acquisition of mDA phenotype, whilst Wnt1 acts as a general midbrain mitogen (Castelo-Branco et al., 2003).

At E8 the mouse homeobox transcription factor *Engrailed-1* (*En1*) and *Engrailed-2* (*En2*) are expressed across the IsO in the caudal midbrain and rostral hindbrain (Simon et al., 2001). In adulthood, En1 and En2 have a more limited expression pattern. En1 is highly expressed by essentially all DA neurons in SN and VTA, whereas En2 is expressed at lower levels and is restricted to a subset of the En1 expressing cells. The striking deletion in the mid-hindbrain junction region from E9.5 in *En1* mutants suggests that En1 is essential for generation and/or survival of mid-hindbrain precursor cells. Yet, mDA neurons are present in *En1* null mice at P0, and En2 expression is up-regulated in these cells (Simon et al., 2001). In *En1/En2* double mutants, TH^+ cells are generated in ventral midbrain at E11 but are smaller and lack of axonal outgrowth. By E14, when almost all wild type mDA neurons express En1, the TH^+ DA neurons disappear in *engrailed* double mutants (Simon et al., 2001). Expression of other mDA markers has not been reported for these TH^+ neurons which are present during E11 to E14, so it is still an open question whether Pitx3 is regulated by En1/En2. The continuous expression of En1/En2 of mDA neurons into adulthood suggests that they may be involved in maintenance of this neuronal population (Alberi et al., 2004; Simon et al., 2001). By using cell-mixing experiments and RNA interference on primary midbrain cell cultures, it has been reported that *En1*/*En2* is required for the survival/maintenance of mDA neurons in a

cell-autonomous manner (Alberi et al., 2004).

The LIM homeodomain transcription factor Lmx1a was shown to be expressed in the ventral midbrain in a spatiotemporal pattern correlating with the onset of mDA neurogenesis (Andersson et al., 2006b). The induction of ventral Lmx1a expression appears to be Shh dependent. Ectopic or overexpression of Lmx1a in the chick neural tube or in ES cells is sufficient to induce mDA neurons in the ventral midbrain or in the presence of Shh, respectively. On the other hand, RNA interference experiments have shown that Lmx1a is required for generation of mDA neurons. In chick, Lmx1a induces Msx1, which in turn suppresses floor-plate characteristics and induces proneural transcription factor neurogenin 2 (Ngn2) for pan-neural differentiation. The basic helix-loop-helix gene Ngn2 is expressed and required by the mDA progenitors for the acquisition of generic neuronal properties (Andersson et al., 2006a; Kele et al., 2006). *Ngn2* null mutation in mice attenuates the generation of mDA precursors, eventually resulting in a reduced number of mDA neurons.

Lmx1b is expressed throughout the mesencephalon and diencephalons from E7.5 in mice and expression is restricted in the SN and VTA from E12.5 onwards (Nunes et al., 2003; Smidt et al., 2000). Studies on *Lmx1b* null mice have revealed that a loss of Lmx1b results in the failure of proper mDA neuron development. At E12.5 there is a reduction in the number of TH expressing cells and an absence of Pitx3 expression in the ventral midbrain of *Lmx1b* null mice. From E16 onwards, TH expression in not detected in the mutant ventral midbrain (Smidt et al., 2000). As Lmx1b is expressed early (E7.5 in mouse) during CNS patterning events, has a widespread expression pattern in the brain, and overexpression studies show that Lmx1b is not a potent

inducer of TH^+ DA neurons (Andersson et al., 2006b), it is difficult to draw conclusions about a direct role for Lmx1b in mDA neurons. Further experiments are needed to address the cell-autonomous functions of Lmx1b in mDA neurons. However, the knockout study suggests that *Nurr1* is not regulated by *Lmx1b* and that *Pitx3* might be a target of *Lmx1b*. Since Lmx1b has been shown to be required for induction and/or maintenance of Wnt1 expression in the chick IsO, it has been suggested that the action of Lmx1b on *Pitx3* may be mediated by the secreted factor Wnt1 (Prakash et al., 2006; Prakash and Wurst, 2006b).

Nurr1 (also known as Nr4a2) is a member of the orphan nuclear receptor family of transcription factors and is strongly expressed in the ventral mesencephalic flexure from mouse E10.5 onwards (Zetterstrom et al., 1997). *Nurr1* deficient mice lack TH expression in the ventral midbrain from E11.5 (Zetterstrom et al., 1997). A later study has revealed that *Nurr1* deficient neuroepithelial cells undergo normal ventralisation and differentiate into Pitx3 expressing neurons (Saucedo-Cardenas et al., 1998). Further studies have demonstrated the expression of En1, En2, AHD2 and AADC in DA cells in *Nurr1* mutant midbrains, although the expression of these markers is reduced or absent by E15.5 (Smits et al., 2003; Wallen et al., 1999). These *Nurr1* deficient midbrain neurons do not express DA neuron markers such as TH, vesicular monoamine transporter 2 (VMAT2) and dopamine transporter (DAT) (Smits et al., 2003) and subsequently die by apoptosis, resulting in a severe loss of SN and VTA cells by neonate stage. Thus, Nurr1 is required for the transcriptional activation of the genes encoding those proteins that confer DA neurotransmitter properties and is necessary for the survival of late dopaminergic neurons (Saucedo-Cardenas et al., 1998).

The first post-mitotic DA neurons are detected at E10.5 in the mouse, characterised by expression of Nurr1 followed by TH expression at E11.5. Terminal differentiation and maturation of an mDA neuron includes expression of all enzymes and transporters required for the proper synthesis, storage, release and reuptake of dopamine, as well as the proper innervation of their target field in the forebrain (Burbach et al., 2003; Wallen and Perlmann, 2003).

- Parkinson's disease

PD is the second most common neurodegenerative disease and affects 1% of population over 50 years of age. The three cardinal clinical features are resting tremors, a slowness of movement (bradykinesia) and a difficulty in initiating movement (akinesia) (Samii et al., 2004). The pathological findings of PD show that mDA neurons in SN are greatly diminished. In some cases the remaining neurons contain intracytoplasmic inclusions known as Lewy bodies. Lewy bodies contain various proteins, including α-synuclein and ubiquitin that might have been defined for degradation but are not broken down. In the striatum there is a loss of dopamine, its metabolites and the dopamine transporters. Clinical signs of PD are evident when about 80% of striatal dopamine and 50% of nigral neurons are lost (Fearnley and Lees, 1991). The observation that massive DA denervation and depletion occurs before even the earliest features of PD appear has been explained by compensatory mechanisms both presynaptically and postsynaptically in the nigrostriatal DA system.

The specific aetiology of PD is unknown and this has made it difficult to develop ideal methods for studying the disease. Age is the most important risk. Other aetiology is likely heterogeneous, with genetic factors predominating in some cases,

and environmental factor in others. For instance, some cases with PD are associated with exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is highly lipophilic and readily crosses the blood-brain barrier, concentrate selectively in mitochondria of mDA neurons and inhibits complex I of the electron transport chain. The single gene abnormalities identified thus far, including *α-synuclein* and *parkin*, cause very few cases. However, the discovery of these genes and their products has already expanded our understanding of the potential mechanisms of neurodegeneration in both familial and sporadic PD.

Since loss of mDA neurons with a reduction of striatal dopamine is possibly the cause of abnormal motor control, pharmacologic dopamine replacement with L-dopa (L-3,4-dioxyphenylalamine), which is the precursor of dopamine, is the mainstay treatment of PD (Mercuri and Bernardi, 2005). Therapy with L-dopa is normally administered in combination with a peripheral decarboxylase inhibitor beserazide or carbidopa to prevent peripheral conversion to dopamine. Other treatment includes intake of dopamine agonists which have a direct action on post-mitotic DA receptors to improve PD symptoms (Thobois et al., 2005). Ablative surgical treatment includes pallidotomy, thalamotomy and, more recently, subthalamotomy. The other surgical alternative is deep brain stimulation of the subthalamic nuclei, thalamus, and internal globus pallidus (Nutt et al., 2003). These treatments are not ideal and sometimes give rise to side effects, such as abnormal movement (dyskinesia) by L-dopa or cognitive disturbance by deep brain stimulation. Therefore, new strategies need to be developed.

Neurotrophic factors, such as glial cell-derived neurotrophicc factor (GDNF), have been proved to increase survival of mDA neurons in cellular and animal models of PD. However, a multi-centre, double-blind, placebo-controlled trial of intraventricular infusion of GDNF failed to confirm clinical benefit at 8 months (Nutt et al., 2003). It has been suggested that the reason of the clinical failure is the method of trophic factor delivery. Another approach to introduce GDNF is by use of the strategy of gene therapy. Kordower et al. have shown neuroprotection by lentiviral vector deliver of GDNF in primate models of PD (Kordower, 2003; Kordower et al., 2000). In addition to GDNF, Bankiewicz et al. have used recombinant adeno-associated virus to deliver aromatic L-amino acid decarboxylase (AADC) gene, which is an important enzyme that converts L-dopa to dopamine, thus reducing L-dopa requirement in animal models of PD and expending the duration during which clinical benefit of the medication is observed (Bankiewicz et al., 2000; Sanftner et al., 2005). During et al. have used adeno-associated viral vectors to deliver glutamic acid decarboxylase (GAD), which synthesises the major inhibitory neurotransmitter GABA, into excitatory glutamateric neurons of subthalamic nucleus. This changes these cells from excitatory to inhibitory and reduces the excessive output from subthalamic nucleus, which is associated with motor dysfunction in patients of PD (During et al., 2001; Luo et al., 2002). Another candidate target for gene therapy under investigation is to block the signalling pathway of apoptosis, thus forestalling neuronal death before irreversible cellular injury (Silva et al., 2005). However, long term gene expression and safety of the strategy of gene therapy need to be established before further clinical trials.

- Cell replacement therapy for PD

The relative simplicity of the major pathology of PD, the loss of a unifocal and phenotypically homogeneous neuron population, have spurred cell-based replacement strategy for PD. Intrastriatal transplantation with human foetal mesencephalic tissue, rich in DA neurons, was started in 1987 with the idea to restore the loss of dopamine release by the impairment of nigrostriatal pathway (Mercuri and Bernardi, 2005). The mesencephalic tissue was taken from aborted human foetus, aged 6-9 weeks after conception. The clinical benefits after foetal mesencephalic transplantation were coupled with an increase in striatal fluorodopa uptake on positron emission tomography (PET) (Kordower et al., 1995). Postmortem studies have confirmed long-term survival of implanted DA neurons with extensive reinnervation of the striatum (Kordower et al., 1995; Kordower et al., 1997). However, two groups had performed prospective, randomised, double-blind, sham surgery-controlled trial of foetal mesencephalic transplantation and both failed to confirm significant clinical benefit despite increased striatal fluorodopa uptake on PET and evidence of surviving transplanted neurons at postmortem (Freed et al., 2001; Olanow et al., 2003).

From available experimental and clinical data, patient selection is a significant factor for determining the functional efficiency of intrastriatal human foetal mesencephalic grafts. Although selective degeneration of mDA neurons is believed to be a common pathology of most PD patients, clinically diagnosed PD patients show a wide range of neuropathology and even extrastriatal changes. In the double-blind, sham surgery-controlled study, Freed et al. have shown clinical benefit in patients younger than 60 years of age, but not in older ones (Freed et al., 2001). Animal studies have

supported that DA-depleted striatum of aged animals is an impoverished environment for survival, growth and function of DA grafts (Collier et al., 1999; Sortwell et al., 2001).

Additionally, it has been suggested that immune rejection after discontinuation of immunosuppressive treatment limits the benefit of transplantation. Long-term immunosuppressive treatment is essential to prevent the development of a delayed immunologic response to the allogeneic graft tissue (Lindvall and Bjorklund, 2004). On the other hand, the successful experience with correct immunosuppression also implicates that therapeutic cloning to avoid graft rejection is not necessary in ES cell replacement therapy for PD.

Furthermore, a critical issue for developing cell replacement therapy is the quantity and quality of transplanted tissue. Survival of embryonic DA neurons is only 3-5% after grafting with current available procedures (Kordower et al., 1995; Kordower et al., 1997), so it is very important to get the right cells with greater survival and better potential for integration into the host brain.

Currently it seems likely that cell replacement therapy will be optimally effective and therapeutically valuable only in moderately advanced PD patients exhibiting a good and reliable response to L-dopa medication in combination with symptomatology and PET image, which suggest intrastriatal dysfunction as the leading cause of disability (Lindvall and Bjorklund, 2004). However, a clinically competitive cell replacement therapy has to provide advantages over current, rather effective treatment in PD patients. So far the improvement after intrastriatal transplant of foetal mDA neurons in PD patients has not exceeded those found with subthalamic

deep brain stimulation and there is no convincing evidence that drug-resistant symptoms are reversed by these grafts (Lindvall and Bjorklund, 2004). Although the results appear to provide proof-of-principle for cell replacement strategy in PD, it is also obvious that further developments are needed before cell-based approaches become clinical treatment.

- The transcription factor Pitx3 in mDA development

In developing mice *Pitx3* mRNA expression in midbrain is first seen at E11.5 as a small layer at the ventral surface of the mesencephalic flexure. This layer corresponds to the first TH expressing cells in the mouse brain. Then the expression is maintained through adult life. In the unilaterally 6-OHDA treated mouse model, Pitx3 expression is no longer detected in the 6-OHDA injection side but is normally expressed contralaterally. In situ hybridisation studies on human brains reveals that PITX3 is expressed in the SN and there is a reduced density of PITX3 expressing neurons in the patients with PD (Semina et al., 1998; Smidt et al., 1997).

Pitx3 expression is strongly hypomorphic in homozygous *aphakia* mice from E11 to newborn (only 5% of wild type level of *Pitx3* transcript at E13.5) (Rieger et al., 2001). Therefore *aphakia* mice are usually used to study midbrain development with loss of Pitx3 protein (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003). The adult *aphakia* mice display loss of TH^+ cells in SN and VTA. Nissl-stained sections of *aphakia* mice have shown that loss of TH immunoreactivity is due to loss of TH^+ cells. In the dorsal striatum where SN DA neurons project, there is also reduced TH immunoreactivity. High pressure liquid chromatography (HPLC) measurement of striatal DA levels is reduced, compared to wild type. Retrograde tracing experiments show that the absence of Fluoro-gold

labelling reflects loss of innervations in the striatum due to neuron loss in SN, rather than a defect in intracellular transport (Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003). These data have shown Pitx3 is not only a specific marker for mDA neurons, but also functionally required for their survival or maintenance.

Reports on the motor function and behaviour phenotype of *Pitx3* hypomorphic *aphakia* mice are inconsistent. In the report of van den Munckhof et al., *aphakia* mice have shown 50~70% reduction in ambulatory and stereotypic activities and spend more time resting compared to wild type (van den Munckhof et al., 2003). In contrast, Nunes et al. have shown that general locomotor behaviour appears to be higher in *aphakia* as compared to wild type (Nunes et al., 2003). Smidt et al. have compared behavioural characteristics of *aphakia* mice with wild type mice, which had been made blind on postnatal day 6-7. They did not find altered posture, walking pattern or tremor, but the mice have lower overall activity in an open field test and increased activity in a climbing test (Smidt et al., 2004). Hwang et al. have selected tests that do not require visual clues and found no deficits in neurological reflex and sensorimotor response (Hwang et al., 2003). It has been suggested that the lack of gross neurological deficits may be due to compensatory mechanism during development.

- *Pitx3*-GFP reporter mice for developmental study of mDA neurons

Prior to the start of this PhD thesis, our laboratory has created mice with an enhanced GFP (eGFP) reporter targeted to the *Pitx3* locus via homologous recombination in ES cells (Zhao et al., 2004). The *Pitx3* DNA sequence, including part of intron 1, exon 2, 3, and part of exon 4, has been replaced with an eGFP*irespac* cassette downstream of the

endogenous *Pitx3* promoter (Figure 1-1 C). This removes the entire *Pitx3* coding sequence, so there is no expression of Pitx3 from the mutated allele, while eGFP expression is under the control of the endogenous *Pitx3* regulatory elements. Mice that are heterozygous and homozygous for the *Pitx3*-GFP allele have been generated from these ES cells and they are able to survive to adulthood, whilst the homozygous *Pitx3* null mice appear to be small and have reduced fertility.

The heterozygous *Pitx3*$^{GFP/+}$ mice have no apparent phenotypic abnormalities. It has been confirmed that in the transgenic embryos and adult mice heterozygous for the *Pitx3*-GFP allele the *Pitx3*-GFP is an accurate reporter of Pitx3 expression. In embryos *Pitx3*-GFP expression is observed in midbrain, eyes, somites, cranial facial muscles, and the tongue, which correlates with reported sites of *Pitx3* mRNA expression (Semina et al., 2000; Semina et al., 1997; Smidt et al., 1997). Furthermore, in adult mice *Pitx3*-GFP is co-expressed with TH in virtually all mDA neurons of the SN and VTA (Maxwell et al., 2005). Therefore, *Pitx3*-GFP can be used as a lineage marker to track the fate of Pitx3 expressing cells in both *Pitx3* heterozygous and *Pitx3* null mice.

In fact, the *Pitx3*-GFP reporter has been used successfully for identification of ontogenetically distinct subgroups of mDA neurons in early stage of development (Maxwell et al., 2005). Some mDA neurons in the midbrain at E12.5 express TH but not Pitx3, whilst another population of cells express Pitx3 but not TH and these two populations of cells partially overlap so that some cells express both TH and Pitx3. As development progress more midbrain cells express both TH and Pitx3 and by adulthood virtually all mDA cells of SN and VTA express both *Pitx3*-GFP and TH.

Mice homozygous for the *Pitx3*-GFP allele, *Pitx3*$^{GFP/GFP}$, are true *Pitx3* null mice which can be used to analyse the functional role of the transcription factor Pitx3 in midbrain and lens development. It has been shown that there is a progressive loss of mDA neurons in *Pitx3* null mice which is restricted to the SN (Maxwell et al., 2005). By E14.5 there is around 60% loss of *Pitx3*-GFP^{+}TH^{+} cells in the SN of *Pitx3*$^{GFP/GFP}$ mice. Eventually, by adulthood a complete loss of *Pitx3*-GFP^{+}TH^{+} neurons is observed in the SN of *Pitx3* null mice. The *Pitx3*$^{GFP/GFP}$ mouse model also provides biological evidence that Pitx3 is required for TH expression in mDA neurons. At E14.5 there is a loss of TH expression in *Pitx3*-GFP positive cells in *Pitx3* null mice and this loss of TH expression is restricted to the SN. The data demonstrate that Pitx3 is not only required for the survival mDA neurons but it is also involved in the regulation of TH expression in a subset of mDA neurons (Maxwell et al., 2005).

The *Pitx3* null mice, as well as the *Pitx3* hypomorphic *aphakia* mice, seem to be a favourable animal model of PD due to a selective and significant loss of SN mDA neurons. However, the rate of mDA neuron loss and the presumed behavioural phenotype of the *Pitx3* null mice do not correlate well with PD. The lack of a severe behaviour phenotype in *Pitx3* null mice can be due to a compensatory mechanism as a result of the early developmental loss of the SN mDA neurons. Therefore, a transgenic mouse, in which *Pitx3* is conditionally knocked out at a later stage in life, may provide further information about the functional role of the transcription factor Pitx3 in the maintenance of mDA neurons and thus may represent a better model of PD.

1.3.2 Embryonic stem cells

- Key features of ES cells

Directly isolated from the inner cell mass of the blastocyst embryo, ES cells are unique stem cells as they retain the developmental potency of foetal founder cells even after extended propagation and manipulation in culture. When transferred to a pre-implantation mouse embryo, ES cells incorporate into the inner cell mass and generate mice that are chimeric both in somatic and germ tissues (Bradley et al., 1984).

ES cells were initially isolated and maintained by coculture on feeder layers of mitotically inactivated mouse fibroblasts (Evans and Kaufman, 1981; Martin, 1981). It was later identified that the fibroblast feeders express the stem cell regulator leukaemia inhibitory factor (LIF) which actively suppresses differentiation (Gearing et al., 1987; Smith et al., 1988). LIF is able to completely replace feeder layers, not only in the maintenance of previously established ES cell lines, but also in the *de novo* establishment of karyotypically normal and germ-line competent ES cell lines (Smith et al., 1988). This property has enabled the culture of a homogeneous population of pluripotent ES cells in the absence of contaminating fibroblasts. Recently, it has been discovered that bone morphogenetic proteins (BMPs) act in combination with LIF to sustain self-renewal and preserve multilineage differentiation of ES cells in serum-free condition (Ying et al., 2003b). This fully defined culture paradigm also supports the generation of germ-line competent ES cell lines. In the absence of LIF, however, BMP stimulates differentiation, suggesting that a very delicate balance of different signalling pathways regulate ES cell self-renewal versus differentiation.

The ES cell genome can be manipulated at will, either via introduction of new genetic information or the alteration of the host gene sequences. The capability of germ-line colonisation means that ES cells can be exploited as vehicles for transgenic manipulation of the mouse genome. Indeed the major use of ES cells in the last decade has been as a cellular tool for the production of mice carrying a predetermined genetic modification generated by homologous recombination or gene targeting. The planned alteration of a gene is first generated in the genome of ES cells in tissue culture. Genetically modified ES cells can then be injected into recipient blastocysts, where they contribute differentiated progeny to their host, resulting in the birth of genetically modified chimeric pups. Following germ-line transmission, mice that carry a defined mutation of a gene are generated. Genetic modifications can now be designed in a sophisticated manner such that the mutation can be temporally and spatially regulated (conditional knockout), by exploiting the Cre-loxP system and cell/tissue specific regulatory elements. Over the past decade, gene targeting by homologous recombination has revolutionised the field of mouse genetics and allowed the analysis of diverse aspects of gene function in vivo.

However, as it will be discussed below, the application of ES cells is not limited to the generation of designer mouse mutations. With the combination of genetic manipulation in ES cells and in vitro differentiation into somatic cell types, ES cells can provide a powerful system to identify key cell fate determinants. ES cells can also serve as a research tool to model diseases and hence to screen for therapeutic drugs as well as to be used directly as future regenerative medicine in cell replacement therapies.

- Prospects for ES cells in modelling disease and drug development

Screening therapeutic compounds with a cellular platform can significantly reduce cost and the usage of animals. There are several DA cell lines available for academic research and commercial drug development. However, these cells are of tumour origin (either derived directly from neuroblastomas or fusion of primary midbrain cells with a tumour cell line) and thus may not respond to drugs in a physiologically relevant manner. The ability of ES cells to produce biologically compatible DA neurons of midbrain phenotype suggests that they could serve as a good cellular system to screen for chemical compounds that support the function or survival of DA neurons. ES cell derivative could also be used for toxicity testing of substances potentially harmful to DA neurons. The feasibility of this approach has been demonstrated by several studies (Aubert et al., 2002; Aubert et al., 2003; Buesen et al., 2004; Ding et al., 2003; Seiler et al., 2004).

Furthermore, genetic manipulation in ES cells provides opportunities to model aspects of human disease in an easily accessible cell culture system (Wilmut, 2004). For example, gene mutations implicated in PD can be engineered in ES cells, either via homologous recombination (gene targeting) or transgene expression. These 'disease' ES cells can then be directed to neural stem cells and/or DA neurons in the laboratory and be used to identify compounds that target particular cellular activities or signalling pathways that are relevant to PD (Ho and Li, 2006).

- ES cells as cell replacement therapy

The relative simplicity of the major pathology of PD, the loss of a unifocal and phenotypically homogeneous neuron population, spurred research into a cell-based replacement strategy for PD. Despite challenges and controversies (Bjorklund et al.,

2003; Dunnett et al., 2001; Freed et al., 2000; Freed et al., 2001; Olanow et al., 2003), foetal transplantation has alleviated symptoms in some PD patients for many years and the idea of further developing cell therapy to replace or rescue degenerated DA neurons has remained promising (Bjorklund et al., 2003; Lindvall and Bjorklund, 2004). It is also evident in animal studies that neuroprotective or trophic molecules which are delivered either via cell grafting or viral vectors can prolong the life of the damaged DA neurons. A key issue is the development of a reliable cell source with well characterised properties.

The ability to produce unlimited amounts of well-defined somatic cell types makes ES cells an obvious candidate for cell therapy. Furthermore, by exploiting genetic manipulation, ES cells can be used as a vehicle to deliver therapeutic genes while at the same time serving as donor cells for transplantation. To realise the potential of ES cells as regenerative medicine for PD, it is essential to establish the type and developmental/maturation stage of cells that are required for functional repair in Parkinsonian brains. Although ES cells might have greater developmental plasticity to respond to host environmental inductive cues that guide DA differentiation, the adult striatum is probably unlikely to retain all instructive signals for DA development from ES cells. Therefore, pre-differentiation of ES cells into neural cells of a certain maturation stage might be the answer (Kuhn and Svendsen, 1999). However, since it is not known which ventral midbrain cell population is specifically required for functional reconstitution in Parkinson's disease brain, it remains an open question how far ES cells will have to be pushed along the neural differentiation pathway *in vitro* before transplantation. Systematic transplantation studies using purified, molecularly defined cell populations from the developing midbrain should

address this important question. Work in this direction is likely to benefit from developmental studies on normal development of the midbrain DA lineage and the molecular make up of cells at each step of DA lineage differentiation.

Understanding the molecular identity of the desired 'donor' cells is essential as one could design a strategy for selecting these cells from heterogeneous populations of differentiated ES cell progeny, and to guide the development of novel approaches for producing the right kind of therapeutic cells. It should be noted however, that phenotypically and functionally satisfying therapeutic cell types may not necessarily need to be derived through normal developmental pathways (Collins et al., 2005).

1.3.3 In vitro differentiation of ES cells to mDA neurons

As an integral part of pluripotency, ES cell differentiation in vitro is heterogeneous in nature. Currently, our abilities to direct ES cells into specific pathways and then to support the viability and maturation of individual differentiated phenotypes in vitro remains limited. Consequently, the differentiated ES cell cultures constitute heterogeneous types of differentiated ES cell progeny with unknown phenotypes. In the absence of knowledge to instruct ES cells into a specific fate, strategies have been developed to enrich or isolate cell phenotypes of interest from the mixed cell population. This can be achieved through selective culture conditioning or fluorescence-activated cell sorting (FACS). In addition, drug resistance or cell sorting capacity conferred by genetic manipulation in ES cells provides another way of isolating a particular cell lineage or cell types (Li et al., 1998a). Using this approach, researchers can successfully label and isolate neural progenitors, neurons and oligodendrocytes from differentiating ES cell cultures. While neural progenitors and

neurons in general can be derived from ES cells with relatively high frequency, the production of a particular neuronal subtype is less efficient.

- DA fate induction by Shh and FGF8

Inspired by previous studies on DA fate induction of naive neuroepithelium by Shh and FGF8 (Ye et al., 1998), Lee et al. have developed a method to produce DA neurons from ES cells using these two factors (Kim et al., 2002; Lee et al., 2000). The protocol starts with a short period of embryoid body (EB) differentiation followed by medium-based enrichment for Nestin positive neural precursor cells (Okabe et al., 1996). The neural precursors are then expanded using FGF2 followed by induction to a DA fate by addition of Shh, FGF8 and ascorbic acid. The authors have reported a production of over 30% TH expressing cells within the neuronal population. These ES cell-derived DA neurons exhibit some electrophysiological properties and release dopamine upon polarisation (Lee et al., 2000). Using a similar approach, Rolletschek et al. have applied an array of survival-promoting factors in the production of DA neurons from ES cells (Rolletschek et al., 2001). They have found that a cocktail containing interleukin (IL)-1β, glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), transforming growth factor (TGF)-β3, and dibutyryl (db)-cAMP enhance RNA expression levels of En1, Mash1, dopamine receptor 2 (D_2R), TH and Nurr1 and increase the proportion of TH positive and DAT positive neurons from ~20 to 40%.

- Stromal coculture system

Kawasaki et al. have identified a stromal cell-derived inducing activity (SDIA) that efficiently induces neural differentiation from ES cells, with a high proportion of TH positive neurons (Kawasaki et al., 2000). This method does not require EB formation

or retinoic acid treatment. They have screened for differentiation of ES cells after coculture with various cell lines and discovered PA6 cells (stromal cells derived from skull bone marrow) to be the best inducer of neuronal differentiation. The nature of SDIA is unclear as PA6 neural inducing activity remains when the cells are fixed thus unable to secrete soluble factors, or when the PA6 cells are separated from the ES cells by a filter. The authors suggest that SDIA may be a secreted factor that is secondarily tethered to the cell surface, as treatment with heparin removes the neural inducing activity.

In the authors' report, 92% of ES cell colonies became $NCAM^+$ neurons. 92% of colonies contained TH^+ neurons. At the cellular level, TH^+ neurons occupied 30% of β-tubulin III^+ neurons, which was 16% of total cell population. In their RT-PCR results, mDA markers Nurr1 and Pitx3 were induced in SDIA-treated ES cells. In culture, these ES cell-derived DA neurons can release dopamine in response to a depolarising stimulus. While they have demonstrated good survival following transplantation to 6-OHDA-lesioned rat Parkinsonian brains, their ability to generate functional repair has not been reported.

- Monolayer neural differentiation method

Ying et al. have reported that neither multi-cellular aggregation via EB formation nor coculture is necessary for ES cells to commit efficiently to a neural fate (Ying et al., 2003b). For monolayer culture, undifferentiated ES cells are dissociated and plated onto gelatinised tissue culture plates in N2B27 medium. TH^+ neurons can be induced with addition of FGF2, Shh and FGF8. However, the regional identity of these TH positive neurons needs to be further investigated (Parmar and Li, in preparation).

- DA neuron differentiation of primate and human ES cells

Using stromal cell coculture system and/or via induction with Shh and FGF8, several groups have reported the production of DA neurons from primate and human ES (hES) cells (Kawasaki et al., 2002; Park et al., 2005; Perrier et al., 2004; Sanchez-Pernaute et al., 2005; Takagi et al., 2005; Yan et al., 2005; Zeng et al., 2004). Furthermore, neurotrophins such as GDNF and foetal midbrain astrocytes can also promote DA neuron production from hES cells (Buytaert-Hoefen et al., 2004; Park et al., 2004). Owing to differences in developmental timing between mice and humans, necessary adjustments and improvement of the differentiation protocols, for example the time window for Shh treatment, have been made to suit hES cells.

Mouse ES cells, when grafted in small numbers, give rise to DA neurons very efficiently in adult rodent brains (Bjorklund et al., 2002; Deacon et al., 1998). Furthermore, transplantation of pre-differentiated mouse ES cells that contain DA neurons provide motor benefits in rodent models of PD (Tabar et al., 2005). It has been shown that hES cell-derived neural precursors can differentiate into neurons, astrocytes and oligodendrocytes following transplantation into normal or lesioned young adult rat brains (Park et al., 2005; Sanchez-Pernaute et al., 2005; Takagi et al., 2005; Zeng et al., 2004). These hES cell progeny also exhibit region-specific differentiation and migration behaviour. However, studies focused on DA neuron differentiation and cell replacement therapy in animal models of PD have generally observed limited behavioural responses with poor cell survival, as compared with those obtained with mesencephalic grafts. These transplantation studies have been carried out using a mixed population of ES cell differentiated progeny which are likely to contain neural cells at different maturation stages, and have non-neural

lineage. The non-neural cells may have adverse effects on the differentiation, survival and migration of DA neurons in the recipient brain. It is also possible that the donor population does not provide the right category of cell types or insufficient numbers. In this respect it is important to identify what the desired cellular phenotype is for grafting in Parkinsonian brain.

1.4 Aims

Prior to the start of this PhD research, *Pitx3*-GFP reporter mice had been created and proved powerful for analysis of the essential roles of the transcription factor Pitx3 in mDA development. The homozygous *Pitx3* mutant mouse is a true *Pitx3* null, and also shows a phenotype characterised by small eyes and no lens. The hypothesis was formed that Pitx3 is essential for lens development. Therefore, the aim of this project was to investigate the functional role of Pitx3 during lens development using the *Pitx3*-GFP reporter mice. Firstly, the lens morphogenesis of heterozygous and homozygous *Pitx3*-GFP reporter mice will be examined. The heterozygous and homozygous *Pitx3*-GFP reporter ES cells which I have created will be used to generate chimeric animals for a fine scale analysis of the cellular change of *Pitx3* null cells during lens development. The *Pitx3* null lens will also be characterised by comparing key molecules or markers associated with the developing lens. These studies lay the foundation for the elucidation of the molecular mechanisms and genetic pathways regulated by Pitx3 during lens development.

Based on the restricted expression pattern of Pitx3 in mDA neurons combined with the finding that *Pitx3*-GFP reporter has proved to be a useful marker to track mDA development in vivo, *Pitx3*-GFP reporter ES cells may be a useful system to investigate mDA development in vitro. Therefore, the aim of the other part of the

project is to develop an ES cell system for functional identification of molecules governing mDA development using the *Pitx3*-GFP reporter. Firstly, the in vitro differentiated *Pitx3*-GFP expressing cells will be further characterised. Then the *Pitx3*-GFP reporter ES cells will be modified to be easy for transgene introduction, or to be able to express transgene in a temporally controlled manner. Homozygous *Pitx3* null ES cells will also be used in differentiation experiments to study mDA development with loss of Pitx3 and to test candidate Pitx3 downstream targets. The final goal is to identify candidate mDA regulators. Although the production of mDA neurons from ES cells draws attentions in the cell replacement therapy for PD patients, what is more important is to define how and when mDA development is regulated and how it extends and progress in the Parkinsonian brain. ES cell-based research may eventually provide necessary information for this insight or for the development of a more effective therapy.

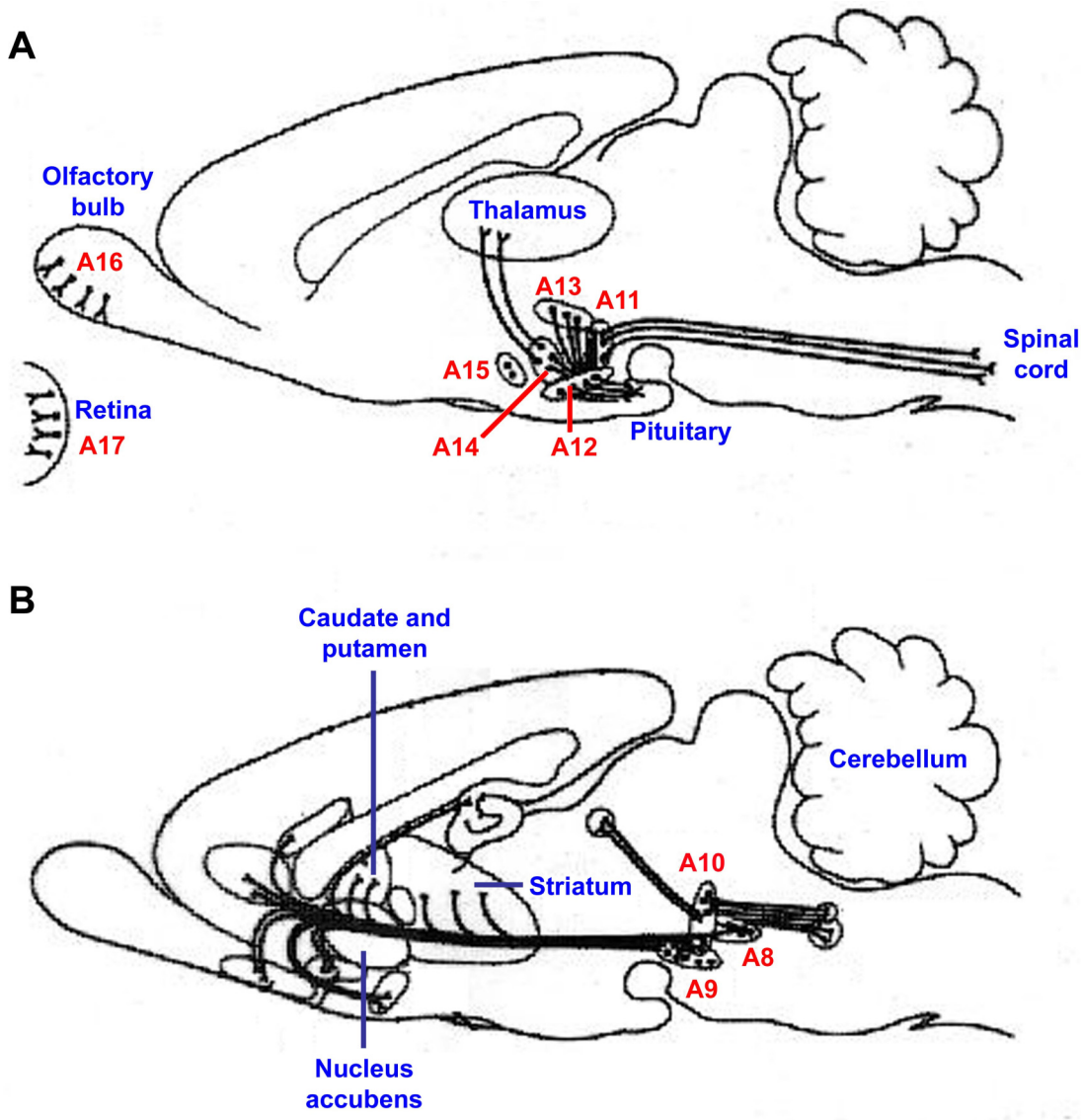

Figure 1-3 Dopaminergic neuron in the brain

(A) Hypothalmic DA neurons in the A11 and A13 cell groups provide descending pathways to the lower brain stem and the spinal cord. Neurons in the A12 and A14 groups are involved with endocrine control. There are also DA neurons in olfactory tubercle (A15), olfactory bulb (A16) and retina (A17). (B) Mesencephalic DA neurons in the ventral midbrain are located in the retrorubral field (A8), the substantia nigra (SN, A9) and ventral tegmental area (VTA). The VTA DA neurons project to limbic structure such as nucleus accumbens. The SN DA neurons project into dorsal striatum forming the nigrostriatal pathway.

Chapter 2

Materials and methods

2.1 Materials

Unless otherwise stated, analytical grade chemicals were obtained from either Sigma or BDH Laboratory Supplies. Stock solutions were prepared with reverse osmosis purified (ROP) water and filtered or autoclaved as necessary. Agarose for electrophoresis was supplied by Invitrogen. Synthetic oligonucleotides were synthesised by Eurogentic SA.

2.1.1 Solutions

Phosphate buffered saline (PBS):

2.7mM KCl, 137mM NaCl, 4.3mM $Na_2HPO_4.7H_2O$, 1.4mM KH_2PO_4 (pH 7.4)

PBST:

0.2% (v/v) Triton X-100 (BDH) and 1% (w/v) bovine serum albumin (BSA, Sigma) in PBS

1X TAE

0.04M Tris-acetate, 0.001M EDTA

20X SSC (Standard sodium citrate)

3M NaCl, 0.3M Na_3Citrate

Lysis buffer:

10mM Tris (pH 8.0), 50mM EDTA, 100mM NaCl, 0.5% SDS, 0.5mg/ml proteinase K (Roche) added just before use.

Luria-Bertani (LB) broth:

1% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract, 85mM NaCl

LB agar:

1.5% (w/v) agar (Difco) in LB broth

MOPS (10X):

0.2M MOPS (pH 7.0), 20mM sodium acetate, 10mM EDTA (pH 8.0)

TFB1:

30mM potassium acetate, 10mM $CaCl_2$, 50mM RbCl, 15% glycerol, pH adjusted to 5.8 with 1M acetic acid and filter sterilised.

TFB2:

10mM MOPS, 75mM $CaCl_2$, 10mM RbCl, 15% glycerol, pH adjusted to 6.5 with 1M KOH and filter sterilised.

PB1 (phosphate-buffered medium 1)

The following were added in ultra high pressure (UHP) water to make a final volume 520 ml PB1 solution: NaCl 4.11g, KCl 0.105g, Na_3PO_4 1.5g, KH_2PO_4 0.1g, glucose 0.52g, sodium pyruvate 0.023g, penicillin 0.03g, $CaCl_2$ 0.6g, $MgCl_2$ 0.5g, 0.5% phenol red 1ml. pH adjusted to 7.0 and sterilised with 0.22 micron bottle-top filter.

The following solutions are for in situ hybridisation.

Prehybridisation mix

50% (v/v) formamide, 5X SSC, 2% Boehringer blocking powder, 0.1% Triton X-1000, 0.5% (w/v) CHAPS, 100ng/ml yeast RNA, 5mM EDTA, 50ng/ml heparin

Solution 1:

50% formamide, 5X SSC, 0.1% Triton X-1000, 0.5% CHAPS

TBT:

50mM Tris-HCl (pH 7.5), 150mM NaCl, 0.1% Triton X-1000

NTM:

100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 50 mM $MgCl_2$

2.2 Molecular biology methods

2.2.1 Cloning

Cloning of DNA fragments was achieved via restriction enzyme digestion, gel purification and ligation. Restriction enzyme digestions were preformed according to manufacturers' instructions (all enzymes were from Roche or NEB). Digested DNA was separated by running on agarose gels (0.6~1.5% depending on size of the fragment) in TAE buffer. The desired fragment was cut from the gel and the DNA extracted using Qiagen gel extraction kit (Qiagen) according to manufacturer's instructions. The concentration of the recovered DNA was estimated by agarose electrophoresis. Ligations were performed with a 3:1 insert:vector molarity ratio with 1U of T4 DNA ligase (Roche) in 1X T4 ligation buffer (Roche) and incubated at

16ºC overnight.

2.2.2 Preparation of chemical competent cells using the rubidium chloride method

A DH5α colony was picked and added to 2.5 ml LB medium and incubated in a shaker at 37ºC overnight. The 2.5 ml culture was added to 250 ml of LB medium with 20 mM $MgSO_4$ and incubated in a 1L flask in a shaker at 37ºC for 5-6 hours, until the $O.D._{600}$ reached 0.4 to 0.6. The 250 ml culture was centrifuged at 4500*g* for 5 minutes at 4ºC. From this stage the protocol was carried out in a room at 4ºC with all equipment chilled. The cell pellet was gently resuspended in 0.4 volumes (100 ml) of ice-cold solution TFB1 and incubated on ice for 5 minutes. The cells were centrifuged at 4500g for 5 minutes at 4ºC. The cell pellet was gently resuspended in 1/25 original culture volume (10ml) of ice-cold solution TFB2. The cells were incubated on ice for 15-60 minutes before aliquotting 200µl per tube and quick-freezing on dry ice. Tubes were then stored at -80ºC.

2.2.3 Transformation of competent cells

A 200µl aliquot of competent cells was thawed on ice. Approximately 10ng of plasmid DNA or ligation reaction was added in 1-3µl of dH2O, gently stirred and incubated on ice for 30 minutes. The tube was heated at 42ºC for 1 minute and then cooled on ice for 2 minutes. 1ml of LB was added to the tube and incubated in a shaker at 37ºC for 1 hour. 50 to 300ul of the culture were plated on LB agar plates with appropriate selection and incubated at 37ºC overnight.

2.2.4 Plasmid isolation from bacteria

To isolate plasmid from bacteria, the following mini-preparation protocol was used. Single colonies were used to inoculate a tube containing 3ml LB medium with

appropriate selection and incubated in a shaker at 37ºC overnight. 1.5ml of the culture was centrifuged at 13000rpm for 3 minutes and the supernatant was discarded. 150µl of solution I was added to the tube and it was vortexed to mix well. To lyse the cells, 150µl of freshly prepared solution II was added and the tube was inverted 4-5 times to mix. To neutralise the lysate, 250µl of cold solution III was added and the tube was inverted 4-5 times to mix. The sample was then centrifuged at 13000rpm for 15 minutes and the supernatant transferred to a clean tube. To precipitate the DNA, 1ml of cold ethanol was added and the tube was inverted 4-5 times to mix before incubating at -20ºC for 30 minutes. The sample was then centrifuged at 13000rpm for 30 minutes to pellet the DNA, which was then dissolved in 50µl dH2O to give a final concentration of about 2.5 µg/ml.

2.2.5 The construct of pCAGEn1IP

The expression vector pCAGEn1IP (Figure 4-10 A, Appendix III) was used to generate En1 overexpression *Pitx3*$^{GFP/+}$ ES cell lines. A plasmid containing *En1* cDNA was kindly provided by Alexandra Joyner (Joyner et al., 1985). A 1.8-kb fragment, including a 1.2-kb length of *En1* cDNA, was cut using the restriction enzyme EcoR1 and recovered. The plasmid pCAGASIP (Appendix I) was linearised using the restriction enzyme EcoR1 as a vector and then phosphorylated with calf intestinal alkaline phosphatase (New England Biolab) to avoid self-ligation. Plasmids were isolated from the transformants and cut by the restriction enzyme EcoR1 to confirm successful insertion, and then checked with the restriction enzyme Cla1 and Sac1 for correct insertion orientation. The correct ligation product should release 2.4- and 5.9-kb fragments by Cla1, and 1.6- and 6.6-kb fragments by Sac1.

2.2.6 The construct pCAGfloxNeoKlf4

To make an inducible *Klf4* expression construct, the *Klf4* cDNA fragment has to be modified with feasible restriction enzyme cutting ends then cloned into our pCAGfloxNeoPA inducible vector (Figure 2-1). Briefly, a 1.7-kb Kpn1-EcoR1 *Klf4* cDNA fragment was cloned from PMT3-Klf4 (Shields et al., 1996) into the pSP72poly4 vector (Appendix VI) in order to create pSP72poly4-Klf4. Then the pSP72poly4-Klf4 plasmid was cut with the restriction enzymes Xho1 and Sal1 to release the *Klf4* fragment which was ligated into the pCAGfloxNeoPA vector that had been cut with Xho1 to create the pCAGfloxNeoKlf4 plasmid (Appendix V).

2.3 Histological analysis

2.3.1 Animal maintenance

Mice were housed and bred in the Biomedical Unit within the Institute for Stem Cell Research, according to the Animals (Scientific Procedures) Act (UK) 1986. Mice were kept in a 12 hours dark, 12 hours light cycle with the midpoint of the dark cycle at 1am. For collection of embryos, matings were set up and female mice were checked daily before 10am for the presence of a vaginal plug. If present then 1pm on that day was designated as embryonic day (E) 0.5. Mice aged 6 weeks or older were used for mating. Litters were left with their mother until 3 weeks of age when they weaned by separating the offspring from the parent. At weaning the mice were sexed and tail tips were taken for genotyping. Maintenance of the mice, breeding set-up and tail biopsies were performed by the Biomedical Unit staff at the Institute for Stem Cell Research.

2.3.2 Cryostat sectioning

Embryos were dissected in PBS, then fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) at 4ºC overnight (for E12.5 and older) or 4 hours (E10.5 to E11.5). The embryos were then cryoprotected by placing them in a solution of 30% sucrose in PBS, which was left at 4ºC until the embryos sink. Embryos were then embedded in OCT (BDH) in foil parcels over dry ice and stored at -80ºC until use. Cryostat sectioning was performed at -16 to -20ºC using a Leica CM 1900 Cryostat and sections were cut at 10μm. Sections were collected on polysine slides (BDH) and allowed to dry at room temperature for 30 minutes and then were either used immediately or stored at -80ºC until use.

2.3.3 Immunochemistry

- For sections

Frozen sections were allowed to equilibrate to room temperature. Sections were blocked with 3% normal serum in PBST for 1 hour at room temperature. Then the sections were incubated with primary antibodies in blocking solution at 4ºC overnight in a humidified box. Sections were washed three times for 20 minutes each in PBST, then incubated with fluorescence-conjugated secondary antibodies at room temperature for 1 hour or overnight at 4ºC, respectively. After washing three times with PBS, sections were mounted in Immunofluore (ICN Biomedicals) and analysed using a Zeiss Axiophot microscope or Leica confocal microscope.

- For cells

For immunohistochemistry, cells were washed twice with PBS and fixed in 4% PFA in PBS for 20 minutes at room temperature. They were then rinsed in PBST twice for

5 minutes to permeabilise the cell membrane, and then incubated in PBST with 3% normal serum (blocking solution) to prevent non-specific binding of the antibody. Then they were incubated in primary antibody at the appropriate dilution in blocking solution overnight at 4ºC. After the incubation, cells were washed 3 times with PBST for 20 minutes and then incubated for 3 hours with the secondary antibody in blocking solution in the dark. Then the secondary antibody was removed and the cells washed 3 times with PBS. The cells can be counterstained with DAPI or Topro for 30 minutes before observing under a microscope.

- Antibody lists

Lens part

Antigen	Species	Supplier	Concentration
β-crystallin	rabbit	Gift from Helen Makarenkova	1:100
γ-crystallin	rabbit	Gift from Helen Makarenkova	1:100
$p27^{KIP1}$	rabbit	Santa Cruz	1:100
$p57^{KIP2}$	rabbit	Abcam	1:100
ECCD2	rat	Developmental Studies Hybridoma Bank	1:100
APA5	rat	Developmental Studies Hybridoma Bank	1:100
pSmad	rabbit	Cell Signalling	1:100
β-catenin	rabbit	Santa Cruz	1:200
Pitx3	rabbit	Gift from M. Smidt	1:200
Pax6	rabbit	Santa Cruz	1:300

ES cell part

Antigen	Species	Supplier	Concentration
GFP	chicken	Chemicon	1:3000
TH	rabbit	Pel-Freeze	1:1000
β-tubulin III	mouse	Babco	1:500
Engrailed-1	rabbit	Developmental Studies Hybridoma Bank	1:100
Nurr1	mouse	Santa Cruz	1:200

Secondary antibody

Conjugant	Antigen	Species	Supplier	Conc.
Alexa Fluoro 488	chicken IgG	goat	Molecular Probes	1:1000
Alexa Fluoro 568	mouse/rat/rabbit IgG	goat	Molecular Probes	1:1000
Alexa Fluoro 647	mouse/rabbit IgG	goat	Molecular Probes	1:1000

Counterstain

Reagent	Supplier	Concentration
DAPI	Molecular Probes	1:1000
Topro	Molecular Probes	1:1000

2.3.4 Image acquisition and manipulation

Coverslips were examined using a Leica TCS SP2 AOBS confocal microscope equipped with krypton, krypton/argon and helium lasers.

2.3.5 Quantitative analysis of immunolabelled cells

To determine the number of antibody-labelled cells in differentiated ES cell cultures, all positively stained cells in a well of a 4-well plate were counted manually. Antibody staining was performed in triplicates, and the data obtained from at least 3 independent experiments were averaged. Numbers shown represented the average percentage and standard error of the mean (SEM). Statistical analysis for the counting data was performed using two sample student *t*-test by Microsoft Excel 2002.

2.4 Cell culture

Generally cells were grown in tissue culture plastics (Iwaki) and incubated at 37ºC with 7% CO_2 in a humidified incubator (Sanyo). All medium and solutions were tested for contamination 3 days before use. When passaging and thawing cells, all medium and solutions were prewarmed to 37ºC. To avoid bacterial and fungal contamination, all tissue culture manipulations were undertaken inside a laminar flow sterile hood (Microflow) and surfaces and objects were sprayed with 70% industrial methylated spirits (IMS) before use.

2.4.1 Culture medium

- Standard culture medium

1X Glasgow minimum essential medium (GMEM, Sigma), 10% (v/v) foetal calf serum (FCS, Gibco), 1mM sodium pyruvate (Gibco), 1X MEM non-essential amino acid (Gibco), 2mM l-glutamine (Gibco), 0.1mM 2-mercaptoethanol (BDH).

- ES cell culture medium

Standard culture medium supplemented with 100 unit/ml leukaemia inhibitory factor

(LIF). LIF was prepared in house by transfecting COS-7 cells with a human LIF expression plasmid and harvesting the medium. The concentration of LIF was assayed using CP1 indicator cells (LIF preparation was performed by Louise Taylor). All medium were tested with established ES cell lines before use.

- ES derivation medium

ES culture medium with 20% FCS and 1% penicillin/streptomycin as antibiotics.

- N2B27 medium

1:1 mixture of DMEM/F12 (Gibco) supplemented with modified N2 (25μg/ml insulin, 100μg/ml apotransferrin, 6ng/ml progesterone, 16μg/ml putrescine, 30nM sodium selenite, 50μg/ml BSA) and Neurobasal medium (Gibco) supplemented with B27 (Gibco).

- Differentiation medium

1X GMEM, 10% (v/v) Knock Out Serum Replacement (KSR, Gibco), 1mM sodium pyruvate, 1X MEM non-essential amino acid, 2mM l-glutamine, 0.1mM 2-mercaptoethanol.

- Induction medium

GMEM medium supplemented with modified N2, 100μM tetrahydrobiopterin (Sigma), 200μM ascorbate (Sigma), 1mM sodium pyruvate, 1X MEM non-essential amino acid, 2mM l-glutamine, 0.1mM 2-mercaptoethanol.

2.4.2 Routine ES cell culture

ES cells were cultured on 0.1% gelatine (Sigma) coated tissue culture flasks or plates in ES cell medium.

- Expansion

To passage the cells the medium was removed using an aspirator then cells were washed twice with PBS. 1X 0.025% trypsin (Invitrogen) was added to just cover the cell monolayer and the flask was incubated at 37ºC for about 3 minutes. The trypsin activity was neutralised by addition of serum-containing medium; 4mls medium was added for every 1ml of 1X trypsin. The cell suspension was transferred to a universal tube (Nunc) and centrifuged for 5 minutes at 1200rpm. Cells were then resuspended in medium to achieve a single cell suspension, counted using a haemocytometer and replated at a density of 10^6 cells per 25cm^2 flask. Routinely, cells were passaged every 2-3 days.

- Freezing

Cells in tissue culture flasks were trypsinised as before and centrifuged for 5 minutes at 1200rpm. Cells were resuspended in 1ml of freezing mix (10% dimethyl sulphoxide (DMSO, BDH) in normal culture medium) per 25cm^2 flask and transferred into a cryotube (Nunc) at 0.5 ml per vial. Cryotubes were placed at -80ºC overnight and then transferred to a liquid nitrogen cell bank (Series 2300, Custom Biogenic Systems).

- Thawing

Frozen cryotubes were retrieved from liquid nitrogen storage and placed immediately into 37ºC waterbath. Once the cells were thawed they were transferred into 9.5ml warm medium to dilute out the DMSO. The cell suspension was then centrifuged for 5 minutes at 1200rpm and subsequently the cells were gently resuspended in 10ml of medium and transferred to a gelatinised 25cm^2 flask. The medium was then refreshed about 10 hours later to remove any dead cells and dilute out the remaining DMSO.

2.4.3 Feeder cell culture

Feeder cells were cultured in standard culture medium on gelatine-free flasks. Passaging, freezing and thawing were as described for ES cells. Before coculture experiments, feeder cells were γ-irradiated to be mitotically inactive then replated into 4-well plates.

Two lines of feeder cells were used in this thesis: mouse embryonic fibroblasts (MEF) for derivation of ES cells from mouse blastocysts, and PA6 stromal cells for in vitro differentiation from ES cells to mDA neurons.

2.4.4 In vitro differentiation

- PA6 coculture system

PA6 stromal cells were irradiated to be mitotically inactive and replated into 4-well plates one day before use. There are 2 steps for in vitro differentiation from ES cells to mDA neurons by PA6 coculture (Kawasaki et al., 2000). ES cell colonies were cultured in differentiation medium at 100 colonies per well in 4-well plates. Medium change was performed on day 4 and every other day following that. The medium was replaced with induction medium on day 8 and then the cells were cultured in induction medium for an additional 8 days.

- Monolayer differentiation

The method used was based on that of Ying et al. (Ying et al., 2003b). Monolayer differentiation of ES cells was performed in 6-well plates (Iwaki) coated with 0.1% gelatin. Routinely, 0.5-1.5x10^4/cm^2 cells were plated onto each well in N2B27 medium. Medium was changed every 2 days.

2.4.5 Introduction of DNA into cells

- Lipofectamine transfection

$1x10^6$ cells were plated onto each well of a 6-well plate. 250µl of serum-free GMEM (prepared as normal but without serum) were mixed with 3µl of Lipofectamine 2000 (Invitrogen) and left at room temperature for 5 minutes. 3µg of DNA in another 250µl serum-free GMEM was then added and the mix left at room temperature for 20 minutes. The DNA/Lipofectamine 2000 mixture was then added to the cells and rocked gently to mix. Then they were left in the incubator overnight. Cells were trypsinised and replated at a density of $5x10^4$ or $2x10^5$ per 10cm culture plate then appropriate selection was added.

- Electroporation of ES cells

ES cells were trypsinised and $2x10^7$ cells pelleted and resuspended in 700µl of PBS. 50µg of linearised DNA resuspended in 100µl of PBS was added to the cells, and the cell-DNA mix transferred to a sterile cuvette (BioRad 0.4cm gap) for electroporation in a BioRad Genepulser electroporator at 0.8kV and 3µF. Immediately after the electroporation, the cells were plated onto 10cm gelatinised tissue culture plates at a density of $2\text{-}5x10^6$ cells per plate. 24 hours after the transfection, the medium was changed and the appropriate selection drug was added. For stable transfection, cells were grown in selection medium for 7-10 days until colonies appeared. Single colonies were picked into gelatinised 24-well plates and then expanded.

Selection drug concentration: G418: 200µg/ml, puromycin 1µg/ml.

2.4.6 Flow cytometry analysis of GFP^+ cells

Cells were trypsinised and resuspended in PBS with 10% FCS at 4ºC and subjected to flow cytofluorometry analysis to assess the percentage of GFP^+ cells. Approximately 10000 cells were analysed per sample using FACSCalibur (Becton Dickison Biosciences) using CellQuest software. Cell debris and some dead cells were excluded from the analysis based on electronic gates set using forward scatter (size) and side scatter (cell complexity) criteria, and then the gated cells were analysed for the proportion of GFP^+ cells in the total population. One line of the undifferentiated *Pitx3*$^{GFP/+}$ ES cells was used as control to set the boundaries.

2.5 Derivation of homozygous *Pitx3*$^{GFP/GFP}$ ES cells from blastocysts

2.5.1 Mouse set up and induction of implantation delay of blastocysts

In order to obtain mutant blastocysts, we set up intercrosses of homozygous *Pitx3* mice with heterozygous ones (on a C57BL/6. genetic background) at a male/female ratio of 1:2. The female mice were examined for the presence of the vaginal plug for 3 days. Noon on the day of finding a vaginal plug was designated embryonic day (E) 0.5. The plugged mice were injected with Depo-Provera 1 mg in PBS subcutaneously and tamoxifen 0.1 mg in propyleneglycol intraperitoneally on E2.5 when the embryos were in the morula stage to delay implantation.

2.5.2 Preparation of flushing medium and flushing pipette

PB1 was used as flushing medium and 10% FCS was added only before use. Pasteur pipettes were finely drawn with appropriate calibration by alcoholic lamp for flushing and transferring cells or embryos.

2.5.3 Flushing delayed blastocysts

All mice were sacrificed just before flushing to keep the viability of blastocysts. Mice were sacrificed on E7.5 with cervical dislocation and the uteri were collected. Under dissecting microscope, the uteri were trimmed to remove adipose and vascular tissue, and the uterine horns were cut to open. The pipette filled with flushing medium was inserted into the uterus through cervical region to flush the uterine cavity. The blastocysts flushed out were collected in an organ culture dish (Nunc), and then transferred to 4-well plates (Nunc) containing irradiated feeder layer, with up to 6 blastocysts per well.

2.5.4 Disaggregration of primary colonies

Disaggregration was performed 5 days after flushing out. The central mass of each explant was picked up from the trophoblasts and washed with PBS twice. Then the ICM-derived components were transferred to separate drops of trypsin. Following incubation at 37ºC for 3 min, the cell mass was gently disaggregated by repeated aspiration through a finely drawn-out Pasteur pipette, preloaded with medium, into smaller cell aggregates of 3-4 cells, and then transferred to 4-well plates with feeder cells in pre-equilibrated complete medium. Each single ICM-derived clump was disaggregated and plated into one single well of a 4-well plate. Medium was changed every other day. When growing into appropriate colony size, the primary ES cell colonies were individually removed, dissociated into single cells using the same procedure and reseeded into 4-well plates with or without feeder cells.

Cells were transferred into 6-well plates, small flasks (25 cm^2) or medium flasks (75 cm^2) then frozen when appropriate. After expansion into small flask, cells were

shifted into normal ES culture medium, which is 10% FCS without antibiotics.

2.5.5 Genotyping

Cells for DNA extraction were cultured in 24-well plates. When confluent, 0.5 ml of lysis buffer with 0.5 mg/ml of proteinase K (Roche) was added into each well and put in the 37ºC incubator overnight. The following day the lysate was transferred to 1.5 ml ependorf tubes and an equal volume of isopropanolol was added to precipitate DNA. DNA was pelleted by centrifugation at 16000 rpm in a benchtop centrifuge (Eppendorf Centrifuge 5415D) for 15 min. After washing with 70% ethanol, the pellet was air-dried for about 10 min and resuspended with 50 µl of sterile water. The DNA solution was put at 4ºC for overnight and then 60ºC water bath for 30 min. After quick spin down, the DNA was ready for PCR reaction.

5 µl of DNA was used for PCR reaction. The primers were O113 (wild type), O114 (mutant) and O115 (common)(Figure 4-2). DNA was initially denatured for 3 min at 94ºC then subjected to 45 cycles of denaturing at 94ºC for 30 sec, annealing at 55ºC for 30 sec and extension at 72ºC for 1 min. After a final elongation step of 10 min at 72ºC, PCR products were analysed by 1.2% agarose gel electrophoresis.

Primer information:

O113: TCCATCGCCGCTTCTATGGT

O114: AGCCTCGACTGTGCCTTCTA

O115: CCGGAGAGGCTGTGAATTAC

2.5.6 Mycoplasma test

Cells for mycoplasma test were cultured in antibiotic free medium for at least 7 days. Two ml of medium was taken and the test was kindly performed by Louise Taylor. In brief, medium samples were used to culture 3T3K cells for 4 days. The medium was removed and 1 acetic acid: 3 methanol was added for fixation. After washing twice with PBS, cells were stained with 500 ng/ml Hoechst stain.

2.6 Diploid aggregation method

The method used was based on that of Nagy. A and Rossant. J (Nagy and Rossant, 1993).

2.6.1 Preparation of aggregation plate

KSOM (Speciality Media) microdrops (roughly 3 mm in diameter) were made in a tissue culture dish (Falcon; Surface Modified Polystyrene) and covered with mineral oil (Sigma). Five depressions were created in each microdrop by pressing a sterilised darning needle into the plastic and making slight circular movement.

2.6.2 Preparation of eight-cell stage wild type embryos

The oviducts along with the upper part of the uterus attached were removed from E2.5 superovulated F1 (CBA x C57BL/6) females and placed into a drop of PB1. Under a dissecting microscope the oviducts are flushed by inserting the flushing needle (Microlance 0.3 x 13mm) attached to a 1 ml syringe of PB1 into the infundibulum. The embryos were collected using a mouth pipette and washed through a drop of PB1 medium to remove any debris.

Groups of embryos (5 max per group) were transferred with as little medium as possible to a drop of Acid Tyrode's in a Petri dish and zona dissolution was carefully observed. As soon as the dissolution was completed the embryos were washed in PB1 drops. Then the zona-free morulae were transferred into aggregation plates by placing one embryo inside each depression.

2.6.3 ES cell/embryo aggregation

ES cells were replated one day before aggregation in order to keep the cells in log phase. On the day of aggregation, one 25cm^2 flask of ES cells were trypsinised and replated on a non-adherent bacterial culture dish and cultured in a 37ºC incubator for 2 hours to allow aggregation formation. Each ES cell aggregate (8-15 cells) was plated in a depression in the microdrop containing a zona-free wild type morula, and cultured in a 37ºC incubator. After overnight culture, the blastocysts formed from aggregates were transferred into the uterine horn of a 2.5 dpc pseudopregnant F1 recipient and the pregnancies were timed according to the pseudopreganat females.

2.7 In situ hybridisation

The method used was based on that of Wilkinson (1992).

2.7.1 Preparation of mouse embryo powder

E14.5 wild type mouse embryos were homogenised with PBS. 4 volumes of ice-cold acetone were added, mixed and incubated on ice for 30 minutes. Then the mixture was centrifuged at 10000g for 10 minutes and the supernatant removed. The pellet was washed with ice-cold acetone and centrifuged again. Then the pellet was ground into a fine powder and allowed to be air dried. The embryo powder was stored at 4ºC.

2.7.2 Preparation of embryos for in situ hybridisation

E10.5 embryos were dissected out in diethyl pyrocarbonate (DEPC, Sigma)-treated PBS and fixed in 4% PFA in PBS at 4ºC overnight. Then embryos were washed twice in PBST at 4ºC then washed with 25%, 50%, 75% methanol/PBST for 20 minutes and stored in 100% methanol at -20ºC.

2.7.3 Riboprobe synthesis

Plasmid DNA was digested with an appropriate restriction enzyme to create a linear DNA template for digoxigenin-labelled riboprobe synthesis. The transcription mixture (50µl) included 1 µg of linearised template DNA, ATP, GTP, and CTP at 1mM each, UTP 0.7mM, digoxigenin-UTP 0.3mM, DTT 10mM, RNase inhibitor (1 unit/µl of transcription mix), and T3 or T7 RNA polymerase (1unit/µl). Transcription was performed for 2 hours at 37ºC. The template DNA was then digested by RNase-free DNase (2µl at 1unit/µl) for 30 minutes at 37 ºC, and all reactions were stopped by addition of 4µl, 250mM EDTA. The riboprobes were then purified by addition of 2.5M NH_4-acetate and 2.5 volume of ethanol. After centrifugation for 30 minutes at 4ºC, the pellet was air dried and resuspended in 100µl DEPC-treated water.

2.7.4 Preabsorption of antibody

Anti-digoxigenin antibody (Roche) was diluted 1 in 1000 in TBT with 10% sheep serum and 2% BSA. Embryo powder was added to the mixture and shaken gently at 4ºC overnight. Then the mixture was centrifuged and the supernatant collected for antibody reaction.

2.7.5 Prehybridisation and hybridisation

Embryos were rehydrated with methanol in PBST in reverse series and washed with PBST 3 times for 10 minutes. Then the E10.5 embryos were treated with 10 μg/ml proteinase K in PBST for 20 minutes and washed with PBST twice for 5 minutes. The embryos were refixed with fresh 0.2% glutaraldehyde/4% PFA in PBST for 20 minutes and washed twice with PBST for 10 minutes. The embryos were treated with prehybridisation mix and allowed to sink. Then they were incubated with fresh prehybridisation mix at 65ºC overnight.

For hybridisation, the embryos were treated with prehybridisation mix with 1 mg/ml digoxigenin-labelled RNA probe, and incubated at 65ºC overnight.

2.7.6 Posthybridisation wash

After hybridization, the embryos were washed at 65ºC with 100% solution 1, 70% solution 1 in 2X SSC and 30% solution 1 in 2X SSC, respectively. Then they were washed with 2X SSC, 0.1% CHAPS twice for 30 minutes, and 0.2X SSC, 0.1% CHAPS twice for 30 minutes, all at 65ºC. After washing with TBT twice for 10 minutes at room temperature, embryos were preblocked with 10% heat-inactivated sheep serum, 2% BSA in TBT for 3 hours at room temperature. Then the embryos were treated with preabsorbed antibody overnight at 4ºC.

2.7.7 Post-antibody wash and colour development

After antibody reaction, embryos were washed 5 times at room temperature for 1 hour then overnight at 4ºC with TBT containing 0.1% BSA. Then embryos were washed twice with TBT for 30 minutes and 3 times with NTM for 10 minutes. Then the embryos were incubated with NTM containing 0.45 mg/ml NBT (4-nitro blue

tetrazolium chloride, Roche) and 0.175 mg/ml BCIP (5-bromo-4-chloro-3-indolyl-phosphate, Roche). When colour had developed to the desired extent, embryos were washed with PBST. The stain was fixed with 4% PFA in PBST at 4ºC overnight.

For sectioning, the fixed embryos were cryopreserved with 30% sucrose and embedded in OCT as described in cryostat sectioning.

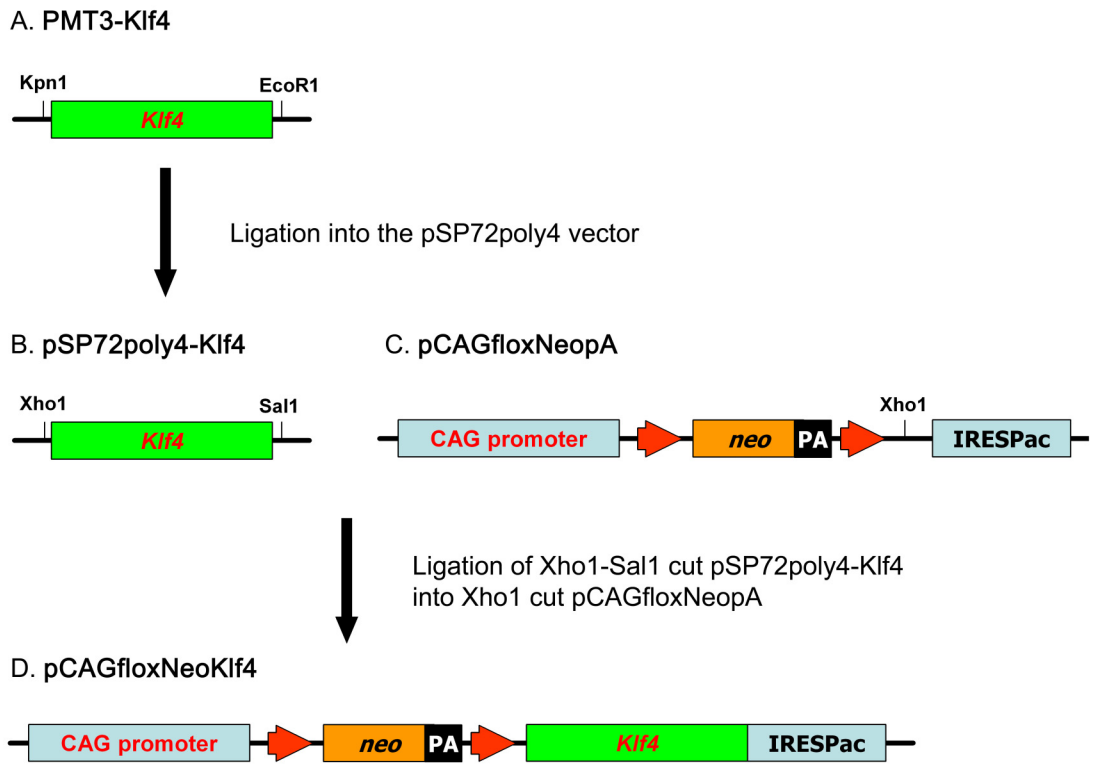

Figure 2-1 pCAGfloxNeoKlf4 vector construction

A 1.7-kb Kpn1-EcoR1 PMT-Klf4 (A) fragment was cloned into a pSP72poly4 vector cut with Kpn1-EcoR1 to create pSP72poly4-Klf4 (B). Subsequently, the pSP72poly4-Klf4 plasmid was cut with Xho1-Sal1 to release the *Klf4* fragment which was then ligated into the pCAGfloxNeoPA vector (C), which had been cut with Xho1, inserting the Klf4 cDNA in between the floxed *neoPA* and the *irespac* to create the pCAGfloxNeoKlf4 plasmid (D). See appendix V for plasmid maps.

Chapter 3

Maintenance of lens epithelial cell property by Pitx3

During the course of my PhD study, the expression and functional role of the transcription factor Pitx3 in midbrain dopaminergic neurons has been investigated by other members of our laboratory and other groups (Hwang et al., 2003; Maxwell et al., 2005; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003; Zhao et al., 2004). *Pitx3*-GFP has been confirmed as an accurate reporter of Pitx3 expression in *Pitx3*-GFP knockin mice, and has been used successfully for identification of ontogenetically distinct subgroups of mDA neurons in early stages of development (Maxwell et al., 2005; Zhao et al., 2004). These studies inspired me to investigate the molecular mechanisms regulated by Pitx3 in lens development using the *Pitx3*-GFP reporter mice and ES cells. (Derivation of the *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ ES cells that were used to produce mouse chimeras is described in detail in Chapter 4.)

3.1 *Pitx3*-GFP expression during lens development

First of all I carried out an investigation into the targeted *Pitx3*-GFP reporter and Pitx3 protein expression during lens development in both wild type and phenotypically normal *Pitx3* heterozygous (*Pitx3*$^{GFP/+}$) mice. The *Pitx3*$^{GFP/+}$ mice showed no abnormality in lens development compared to the wild type littermates. *Pitx3*-GFP was first visualised in E10 embryos when expression was confined to the lens cup. At E10.5, all cells throughout the lens vesicle expressed *Pitx3*-GFP (Figure 3-1 A), which could be easily visualised directly under the fluorescence microscope. Between E11.5 and E17.5 *Pitx3*-GFP expression was observed throughout the lens in both the epithelial and fibrous compartments (E11.5 to E13.5 illustrated in Figure 3-1 B-D). After E14.5, however, Pitx3 protein was primarily found in the lens epithelium and the lens equatorial region where lens epithelial cells exit from cell cycle and

initiate differentiation into fibre cells (Figure 3-2), although *Pitx3*-GFP was still detectable in the lens fibrous compartment, whereas Pitx3 protein was barely detectable.

The physiological relevance of Pitx3 in lens expression is demonstrated by severe developmental lens defects in *Pitx3* null mice. While the lens vesicles were formed in *Pitx3* null embryos by E10.5, they appeared slightly smaller than in the wild type and the *Pitx3* heterozygous control lens (Figure 3-1 E). Furthermore, the *Pitx3* null lens remained attached to the surface ectoderm with a persistent lens stalk. Starting at E11.5, the lens vesicle structure collapsed and lost a regular spherical geometry in comparison with that of the wild type and heterozygous littermates (Figure 3-1 F-H). The lumen was filled with disorganised cells and the *Pitx3*-GFP positive lens remnants did not detach from the surface ectoderm (Figure 3-1 F-G). The lens remnants in the mutant eyes decreased in size as development proceeded. By E15, the lens cells were barely present in the *Pitx3* mutant eye as observed through examination of *Pitx3*-GFP expression under a fluorescence microscope. The lens defect could be detected by directly visualising the GFP reporter expression in a whole embryo. This feature is useful to distinguish the *Pitx3* homozygous embryos from the *Pitx3* heterozygous ones without the need for molecular genotyping (Figure 3-1 D, H).

In contrast to the severe lens defect, the neural retina developed fairly normally with the basic architecture of the neural retinal layer forming (Figure 3-1 A-C, E-G). At later foetal stages following the complete loss of the lens-derived tissue, the *Pitx3* mutant neural retina was folded into the empty chamber. It is likely, therefore, that

the retinal folding in the *Pitx3* deficient eye is secondary to the lens defect.

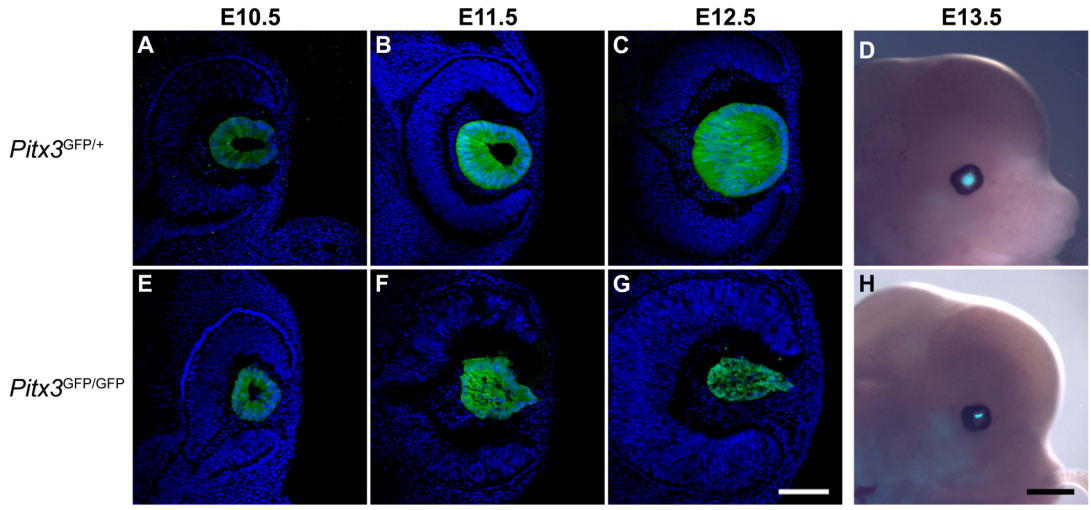

Figure 3-1 *Pitx3*-GFP expression in *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ mice

Pitx3-GFP mirrors the expression of the transcription factor *Pitx3* during lens development (A-D). At E10.5 the *Pitx3*-GFP was expressed throughout all cells of the lens vesicle (A). At E11.5, cells at the posterior part of the lens vesicle began to elongate (B) and occupied the cavity of the lens vesicle at E12.5 (C). In *Pitx3*$^{GFP/GFP}$ mice, the expression of *Pitx3*-GFP throughout the lens vesicle was comparable to *Pitx3*$^{GFP/+}$ lenses at E10.5 (E). The lens vesicle collapsed and filled with disroganised mutant cells after E11.5 (F-H).

Scale bars are 100 µm for A-C, E-G and 1 mm for D, H.

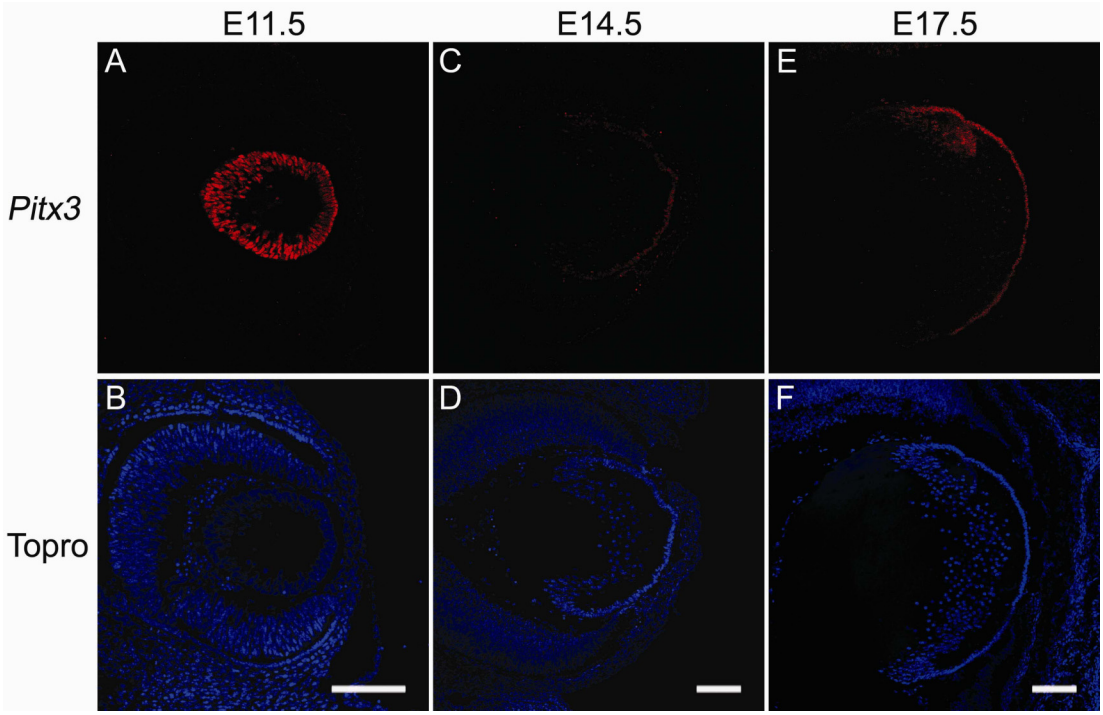

Figure 3-2 Pitx3 expression during lens development

Immunohistochemistry revealed that the transcription factor Pitx3 was expressed throughout cells at E11.5, the lens vesicle stage (A-B). Later, Pitx3 was downregulated after differentiation (C-F).

Scale bars are 100 μm.

3.2 A cell-autonomous requirement of Pitx3 in lens epithelium: a chimeric analysis

The rapid dysmorphogenesis of *Pitx3* null lens at E11.5 and the distorted lens structure precludes a direct investigation on the potential specific requirement of Pitx3 function by lens epithelial or fibre cells. To gain insight into the cellular events underlying deformed lens formation, a series of chimera experiments were performed to provide a fine-scale analysis of the cellular change in *Pitx3* null cells in developing lens.

3.2.1 Introduction for chimeric analysis

A chimera is a composite organism with two or more genetically distinct populations of cells that are derived from more than one individual. Chimera experiments are classic tools to determine whether a gene functions cell-autonomously or non-autonomously, information that cannot be reliably gained by any other means (Collinson et al., 2004; Rossant and Spence, 1998). A cell-autonomous requirement is apparent when the mutant cells exhibit a mutant phenotype within the tissue to which they contribute, or they are excluded from the affected tissue, including depletion of numbers or abnormal distribution. At high contribution in the affected tissues, mutant cells impose a mutant phenotype on surrounding wild type cells. On the other hand, if the mutant cells are defective in a cell non-autonomous function, the presence of wild type cells can rescue the mutant phenotype. By comparing the behaviour of wild type and mutant cells in chimeras, it is possible to assess the ability of mutant cells to contribute to lens tissues and to participate during development when in direct competition with wild type cells.

Previously I have shown that *Pitx3*-GFP expression was fairly uniform in both non-chimeric *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ developing lens. In addition, *Pitx3*-GFP is easy to detect and cell-localised, thus it could be used as a lineage marker to track the distribution of *Pitx3* expressing cells in chimera experiments.

Chimeras can be produced by aggregation of two genetically distinct embryos at the eight-cell stage, or by introducing donor ES cells into recipient embryos via blastocyst injection. In the case of morula aggregation, either embryo to embryo or ES cells to embryo, chimeric embryos are cultured in vitro to the blastocyst stage then they are surgically transferred to the uteri of pseudopregnant fosters for further development.

Before the advances of gene targeting made in ES cells, making chimeras between wild type and homozygous lethal mutants involved the use of embryos derived from heterozygous crosses as one of the chimeric partners. Thus, it was not straightforward to distinguish heterozygote ↔ wild type chimeras from homozygote ↔ wild type combinations, even if a linked marker for the mutation was available. Since *Pitx3* mutation is not lethal and *Pitx3* null morulae or blastocysts are available, it is possible to derive chimeras from early mutant embryos by designed crossmating, without genotyping to distinguish whether the chimeric embryo is derived from heterozygotes or homozygotes. Furthermore, using *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ ES cell lines, which I have established from mutant blastocysts (Chapter 4.2), to generate chimeras, there is no limitation on numbers of mutant embryos available and all offspring are of the required genotypic combination, dramatically simplifying chimeric analysis of mutants.

Pitx3 heterozygous (Pt3A) and *Pitx3* null (Pt2B) ES lines were used to produce *Pitx3*$^{GFP/+}$ ↔ wild type and *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras, respectively. In brief, ES cells were dissociated by trypsinisation and cultured in a non-adherent bacterial culture dish for 2 hours at 37ºC to allow the formation of ES cell aggregates. Each ES cell aggregate (8-15 cells) was then cultured with a zona-free wild type morula overnight. The following day the blastocysts formed from those chimeric embryos were transferred into the uterine horn of a pseudopregnant foster female and collected at E11.5 and E14.5. All aggregations and embryo transfers were performed by Renee MacLay, Ken Jones, Gregor Russel and John Agnew at the ISCR. The embryos in which *Pitx3*$^{GFP/+}$ or *Pitx3*$^{GFP/GFP}$ cells successfully contributed were identified by visualising *Pitx3*-GFP expression in the developing lens under a fluorescence microscope and collected for further analysis.

With the use of eGFP in the heterozygous and homozygous *Pitx3* mutant ES cells, the distribution of donor cells can be easily detected and quantified. The expression of *Pitx3* in the developing tongue is developmentally neutral, that is, *Pitx3* does not influence tongue development. Although the contributions of the donor ES cells in different chimeric embryos varies widely, tissues within an individual chimera usually have a similar composition, so *Pitx3*-GFP distribution in the developing tongue can be used as an indicator to give a global value of the percentage of cells contributed from donor ES cells, and the distribution of mutant cells in the developing lens can be compared.

3.2.2 Underrepresentation of *Pitx3* null cells in lens epithelium

Chimeric embryos were firstly analysed at E11.5. A total of 8 out of 23 and 7 out of 27 embryos collected contained GFP expressing cells in *Pitx3*$^{GFP/GFP}$ ↔ wild type and *Pitx3*$^{GFP/+}$ ↔ wild type group, respectively. All the *Pitx3*-GFP positive cells in the left eye of each chimera were counted to determine their contribution, while the contribution of *Pitx3*-GFP^{+} cells in the tongue (%T) was determined by counting 200 cells. The contribution of *Pitx3*-GFP^{+} cells in the tongue (%T), the lens epithelium (%E), and the lens fibre compartment (%F_{total}) of the *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras was compared to the distribution of *Pitx3*-GFP^{+} cells in *Pitx3*$^{GFP/+}$ ↔ wild type chimeras. These data are presented in Table 3-1 and summarised in Figure 3-5, 3-6.

In *Pitx3*$^{GFP/+}$ ↔ wild type chimeras, GFP^{+} cells contributed fairly randomly to both the epithelial part and the fibre cell part of the E11.5 lens (Figure 3-3 A, C). The E/T ratio (%E over %T) and the F/T ratio (%F_{total} over %T) were calculated separately for each chimera for obtaining a quantitative comparison of the donor cell distribution in different tissues. Both the E/T and F/T ratios were close to 1.0 (Figure 3-5 A-B), demonstrating that the chimerism of these tissue were strongly positively correlated. Therefore, the distribution of these heterozygous *Pitx3*$^{GFP/+}$ cells in both the epithelial and the fibrous compartment was comparable to their chimerism determined by tongue distribution.

In the *Pitx3*$^{GFP/GFP}$ ↔ wild type group, one homozygous chimera with high ES cell contribution (%T=89.1) showed disorganised lens vesicle formation which was similar to that observed in non-chimeric *Pitx3* homozygous mutant lens at E11.5

(Figure 3-3 B). The collapsed lens structure did not detach from the surface ectoderm and the lens cavity was abnormally filled with mutant cells. However, relatively normal lens were formed in moderate and low chimeric *Pitx3*$^{GFP/GFP}$ ↔ wild type embryos (Figure 3-3 D). Interestingly, *Pitx3*$^{GFP/GFP}$ cells were rarely observed in the lens epithelium of these chimeras.

For heterozygous chimeras, the mean (± SEM) of the E/T ratio was 0.84 ± 0.31 (n=7). For homozygous chimeras, the E/T ratio was 0.41 ± 0.23 (n=7), indicating significant underrepresentation of *Pitx3*$^{GFP/GFP}$ cells in the lens epithelium (p<0.02, t-test)(Table 3-1). *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeric embryos provide an environment in which the wild type and *Pitx3* null cells can mix and interact. The underrepresentation of the *Pitx3*$^{GFP/GFP}$ cells in the lens epithelium shows that they cannot be rescued by neighbouring wild type cells and implies that *Pitx3* acts cell-autonomously within the lens epithelium.

The contribution of *Pitx3* mutant cells in the fibrous part of the E11.5 lens was comparable to that of the *Pitx3* heterozygous cells. This was reflected by the F/T ratio of 0.93 ± 0.29 for heterozygous and 1.15 ± 0.37 for homozygous chimeras, respectively (p=0.25). These *Pitx3* mutant cells appeared morphologically normal and were intermingled with differentiating wild type host fibre cells. However, aggregation and physical exclusion of some *Pitx3* mutant cells into the lumen of the lens vesicle was observed (Figure 3-3 D, arrowhead).

3.2.3 Segregation of *Pitx3* mutant cells in lens fibrous part after differentiation

Chimera experiments at E11.5 revealed that *Pitx3* had an autonomous role in the lens epithelial cells. However, *Pitx3* null cells at E11.5 were not eliminated from the

fibrous part although some aggregations of mutant cells were observed. To investigate the developmental potential of these remaining *Pitx3* homozygous mutant cells in the lens fibrous part, E14.5 chimera experiments were performed. A total of 28 and 16 embryos were produced in homozygous and heterozygous groups, and 14 and 5 of them were chimeric embryos with GFP^+ cell distribution, respectively (Table 3-2, Figure 3-5, 3-6).

In the $Pitx3^{GFP/+}$ ↔ wild type embryos, GFP^+ cells were randomly mixed with wild type cells in both the lens epithelium and the fibrous compartment (Figure 3-4 A).

In the $Pitx3^{GFP/GFP}$ ↔ wild type group, three high chimeric (%T >85%) embryos were found to have collapsed lens structure with a persistent lens stalk, showing the same morphology as E14.5 non-chimeric *Pitx3* null lens (Figure 3-4 B). In addition to these 3 high chimeric embryos, another 11 chimeras, of which the chimerisms of $Pitx3^{GFP/GFP}$ cells were mild or moderate, showed formation of lens proper by wild type cells with various degrees of mutant cell distribution (Figure 3-4 C-F).

For heterozygous chimeras, the E/T ratio was 0.93 ± 0.12 (n=5), confirming that $Pitx3^{GFP/+}$ cells contributed equally between the lens epithelium and the developing tongue (Figure 3-4 A). In contrast, for homozygous chimeras, the E/T ratio was 0.0057 ± 0.0134 (n=11). These values were very significantly different ($p<0.0001$, *t*-test)(Table 3-2, Figure 3-5 C), showing that in E14.5 eyes there was a near-complete absence of $Pitx3^{GFP/GFP}$ cells from the lens epithelium of homozygous chimeras (Figure 3-4 C-E). Those $Pitx3^{GFP/GFP}$ cells that had populated the lens epithelium at E11.5 were rarely seen in E14.5 lens. This suggested that proliferation and/or survival capacity of the lens epithelial cells was greatly compromised in the

absence of functional *Pitx3*.

In the fibrous compartment of the homozygous chimeras of mild or moderate chimerism, very few mutant cells differentiated into elongated mature fibre cells (Figure 3-4 C-E). The $\%F_{diff}$, the percentage of elongated differentiated *Pitx3*-GFP^{+} lens fibre cells, was only 0.3 ± 0.8%. However, the fibrous region contained significant proportions of mutant cells, which occurred as segregated patches between the lens epithelium and the lens fibre compartment, or at the posterior edge of the lens proper (Figure 3-4 C-E). In the *Pitx3*$^{GFP/GFP}$ ↔ wild type embryos with moderate chimerism, outside the lens proper there were protruding buds, surrounded by elongated mutant cells, physically excluded from the lens proper (Figure 3-4 D-F). Sometimes a stalk of tissue can be observed to connect the ectopic bud to the lens proper (Figure 3-4 E, arrow). Similar ectopic protruding was never observed in *Pitx3*$^{GFP/+}$ ↔ wild type chimeras. Segregation of cell types suggested that interactions between *Pitx3*$^{GFP/GFP}$ and wild type cells were abnormal so that they failed to mix freely, but actively sorted themselves out into homogenous patches.

To ask the nature of those protruding buds of *Pitx3*$^{GFP/GFP}$ cells from the lens proper, antibody staining of β-crystallin was performed. The strong expression of β-crystallin, a key fibre cell-specific marker, indicated that molecular differentiation has occurred in these *Pitx3*$^{GFP/GFP}$ cells in the fibrous compartment (Figure 3-4 F-G). Although the F/T ratios of the homozygous and heterozygous chimeras were comparable at E11.5 (Figure 3-5 B), the F/T ratio in the *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras (0.34 ± 0.22, n=11) was significantly lower than that of *Pitx3*$^{GFP/+}$ ↔ wild type chimeras (0.97 ± 0.04, n=5, $p<0.0001$)(Figure 3-5 D) at E14.5. The E/T and F/T ratios were not

affected by the chimerism of the mice (%T). However, when the percentage of *Pitx3* null cells was higher than a certain threshold (~85%), the lens development could not be maintained by the wild type host cells and the morphogenesis of the disorganised lens structure resembled that of non-chimeric *Pitx3* null lenses (Table 3-2).

Accordingly, three distinctive classes of embryonic lens arose in the *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras. The first class (dysmorphology I, 4 embryos) includes normal developing lens with segregated patches of *Pitx3* null cells. In the second class (dysmorphology II, 7 embryos) the development of the lens proper was maintained by wild type cells but the ectopic protrusions surrounded by *Pitx3*$^{GFP/GFP}$ cells were found. In the third class (dysmorphology III, 3 embryos), which included a high percentage of *Pitx3*$^{GFP/GFP}$ cells, the wild type host cells could not maintain lens development and the disordered morphogenesis of *Pitx3* null lens was reproduced (Table 3-2).

In summary, while the distributions of the *Pitx3*$^{GFP/+}$ cells in the lens epithelium and the lens fibrous part were comparable to those in the tongue, *Pitx3*$^{GFP/GFP}$ cells were specifically excluded from the lens epithelium of phenotypically rescued developing lens, demonstrating that *Pitx3* is autonomously essential for the development of lens epithelial cells. In the fibrous part at E14.5, most *Pitx3*$^{GFP/GFP}$ cells were not integrated well with wild type fibre cells in the lens proper. They were segregated and even physically excluded from the lens proper although they had shown signs of fibre cell differentiation. This suggests that *Pitx3* may also promote cell surface/adhesion molecule change during fibre cell differentiation (Figure 3-6).

Table 3-1 Analysis of distribution of *Pitx3*$^{GFP/+}$, *Pitx3*$^{GFP/GFP}$ cells in E11.5 chimeric lenses

Chimeras	%T	%E	$\%F_{Total}$	$\%F_{Diff}$	E/T	F/T	dysmorphology
Pitx3$^{GFP/GFP}$ ↔ wild type							
EO2	89.1%						++
EO5	96.1%	2.6%	78.3%	73.5%	0.03	0.82	+
EO3	70.7%	29.6%	73.7%	61.8%	0.42	1.04	+
EO8	63.9%	31.1%	60.3%	57.0%	0.49	0.94	+
EO4	63.7%	27.8%	76.7%	69.3%	0.44	1.20	+
EO7	56.7%	33.3%	55.6%	54.5%	0.59	0.98	+
EO1	46.7%	10.3%	53.5%	53.5%	0.22	1.14	+
EO6	26.3%	18.6%	50.6%	50.6%	0.71	1.93	+
Mean	60.6%	21.9%	64.1%	60.0%	0.41	1.15	
SEM	21.5%	11.7%	11.8%	8.6%	0.23	0.37	
Pitx3$^{GFP/+}$ ↔ wild type							
EE2	96.4%	71.4%	66.2%		0.74	0.69	
EE4	92.1%	82.1%	73.4%		0.89	0.80	
EE8	69.8%	71.4%	76.7%		1.02	1.10	
EE3	68.2%	41.7%	53.6%		0.61	0.79	
EE6	53.0%	26.7%	38.8%		0.50	0.73	
EE5	46.3%	65.2%	70.7%		1.41	1.52	
EE1	31.4%	21.4%	28.8%		0.68	0.92	
Mean	65.3%	54.3%	58.3%		0.84	0.93	
SEM	23.7%	24.1%	18.5%		0.31	0.29	

%T, %E: percentage of GFP^+ cells in the tongue and the lens epithelium, respectively.

$\%F_{Total}$: percentage of all GFP^+ cells in the fibre part, including the integrated and excluded cells.

$\%F_{Diff}$: percentage of GFP^+ cells integrated well with wild type cells in the fibre part.

Lens dysmorphology is shown as: (+) Normal morphology with some mutant cell aggregations; (++) collapsed lens vesicle with a persistent lens stalk.

SEM: standard error of the mean

Table 3-2 Analysis of distribution of *Pitx3*$^{GFP/+}$, *Pitx3*$^{GFP/GFP}$ cells in E14.5 chimeric lenses

Chimeras	%T	%E	$\%F_{Total}$	$\%F_{Diff}$	$\%F_{In}$	E/T	F/T	dysmorphology
Pitx3$^{GFP/GFP}$ ↔ wild type								
LO1	91.5%							III
LO3	85.1%							III
LO7	95.6%							III
LO4	91.0%	0	29.7%	2.7%	8.2%	0	0.33	II
LO2	72.3%	2.9%	41.8%	0.3%	10.3%	0.04	0.58	II
LO12	65.4%	0	15.1%	0	0.4%	0	0.23	II
LO9	63.2%	1.4%	9.5%	0	0	0.02	0.15	II
LO10	51.9%	0	15.6%	0	10.7%	0	0.30	II
LO8	48.2%	0	8.4%	0.6%	8.4%	0	0.17	I
LO14	36.3%	0	18.2%	0	12.8%	0	0.50	II
LO11	35.3%	0	3.0%	0	3.0%	0	0.08	I
LO3	33.5%	0	5.1%	0	5.1%	0	0.15	I
LO5	32.8%	0	14.4%	0	14.4%	0	0.44	I
LO6	22.2%	0	17.4%	0	12.9%	0	0.78	II
Mean	50.2%	0.4%	16.2%	0.3%	7.8%	0.0057	0.339	
SEM	20.8%	0.9%	11.2%	0.8%	5.1%	0.0134	0.217	
Pitx3$^{GFP/+}$ ↔ wild type								
LE3	96.6%	80.6%	93.4%			0.83	0.97	
LE1	76.3%	70.3%	78.8%			0.92	1.03	
LE2	70.0%	71.1%	68.9%			1.02	0.98	
LE5	53.5%	42.8%	50.5%			0.80	0.94	
LE4	33.0%	35.6%	31.1%			1.08	0.94	
Mean	65.9%	60.1%	64.5%			0.93	0.97	
SEM	24.0%	19.7%	24.3%			0.12	0.04	

%T, %E: percentage of GFP^+ cells in the tongue and the lens epithelium, respectively.

$\%F_{Total}$: percentage of all GFP^+ cells in the fibre part, including those fully

differentiated elongated fibre, segregated patches and ectopic buddings.

$\%F_{Diff}$: percentage of elongated differentiated GFP^+ fibre cells in the fibre part.

$\%F_{In}$: percentage of GFP^+ cells kept in the lens proper, including differentiated cells and segregated patches.

Lens dysmorphology is shown as: (I) Normal morphology with some mutant cell aggregations; (II) normal lens proper with ectopic buddings; (III) club shaped lens remnant with a persistent lens stalk.

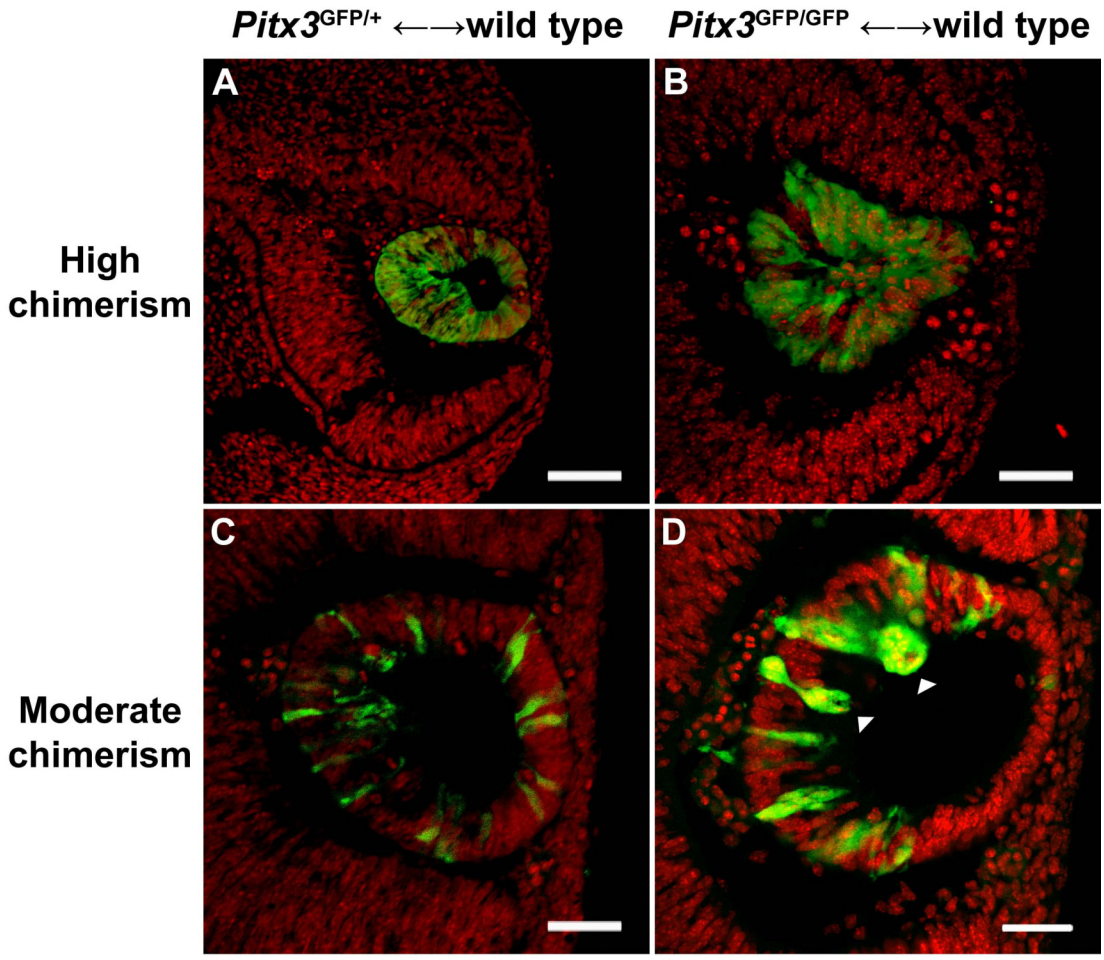

Figure 3-3 Lens of chimeras derived from *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ ↔ wild type embryos at E11.5

In *Pitx3*$^{GFP/+}$ ↔ wild type embryos, *Pitx3*$^{GFP/+}$ cells contributed randomly in both the epithelial and fibre part (A, C). In *Pitx3*$^{GFP/GFP}$ ↔ wild type embryos with high contribution of *Pitx3* null cells, there was disorganised lens development with persistent lens stalk (B). In lower contribution chimeric embryos, there was underrepresentation of *Pitx3* null cells in the lens epithelium (D), suggesting *Pitx3* was autonomously required for lens epithelial cells. In the fibre part *Pitx3* null cells differentiated into elongated fibre cells, but some *Pitx3* mutant cells began to be physically excluded from the lens proper (D, arrowhead).

Scale bars are 80 μm for A and 40 μm for B-D.

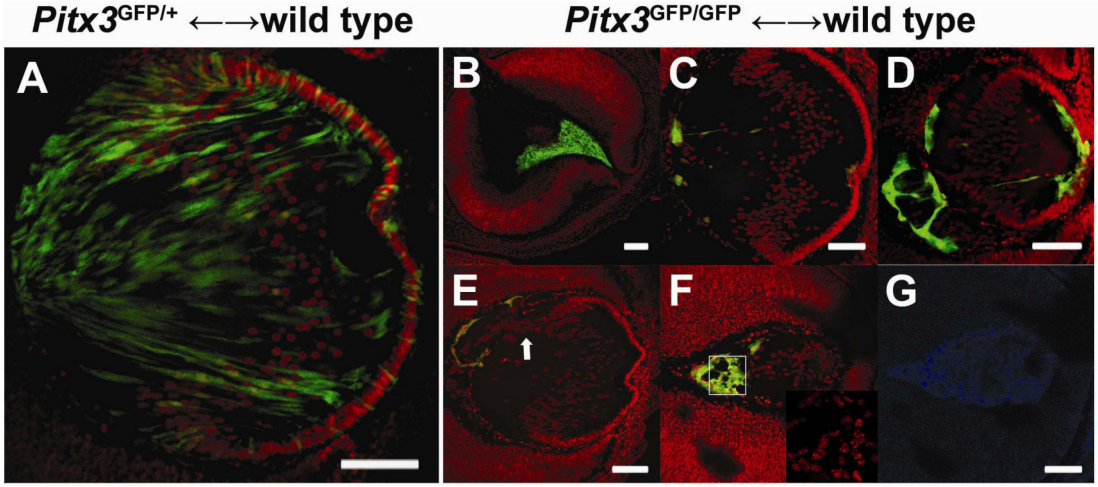

Figure 3-4 Lens of chimeras derived from *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ ↔ wild type embryos at E14.5

In *Pitx3*$^{GFP/+}$ ↔ wild type embryos, *Pitx3*$^{GFP/+}$ cells contributed randomly in both the epithelial and fibrous part (A). In *Pitx3*$^{GFP/GFP}$ ↔ wild type embryos with high contribution of *Pitx3* null cells, there was disorganised lens development with persistent lens stalk (B). In lower contribution chimeric embryos, *Pitx3*$^{GFP/GFP}$ cells rarely contributed to the lens epithelium (C-E). Few *Pitx3* null cells differentiated into mature elongated fibre cells. Most *Pitx3*$^{GFP/GFP}$ cells were segregated from wild type cells (C-E, arrow: a stalk connected to the lens proper). The excluded *Pitx3*$^{GFP/GFP}$ cells expressed β-crystallin (G: β-crystallin staining of F) showing differentiation property. Nuclear morphology of the excluded *Pitx3*$^{GFP/GFP}$ cells revealed no signs of apoptosis (inset of F).

Scale bars are 80 μM.

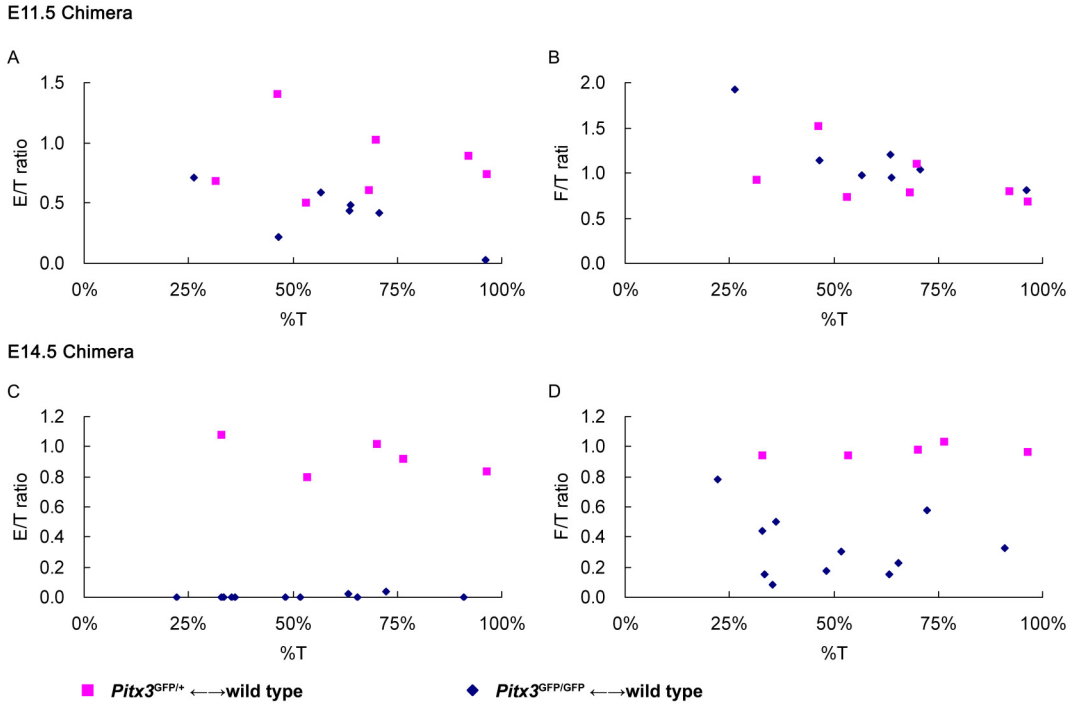

Figure 3-5 E/T and F/T ratios of *Pitx3*$^{GFP/+}$ ↔ wild type and *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras

The percentage of GFP^{+} cells in the lens epithelium or the lens fibrous part was divided by the percentage of GFP^{+} cells in the tongue (%T) for each of the chimeric animals and the ratios E/T and F/T were plotted against %T. The E/T and F/T ratios of *Pitx3*$^{GFP/+}$ ↔ wild type chimeras were around 1.0. Analysis at E11.5 showed that *Pitx3* null cells tended to be eliminated from the lens epithelium (A), while *Pitx3*$^{GFP/GFP}$ cells still remained in the fibrous part (B). By E14.5, the elimination of *Pitx3*$^{GFP/GFP}$ cells from the lens epithelium was almost complete (C), and *Pitx3*$^{GFP/GFP}$ cells were also underrepresented at the fibrous part (D). The E/T and F/T ratios were not affected by %T at E14.5.

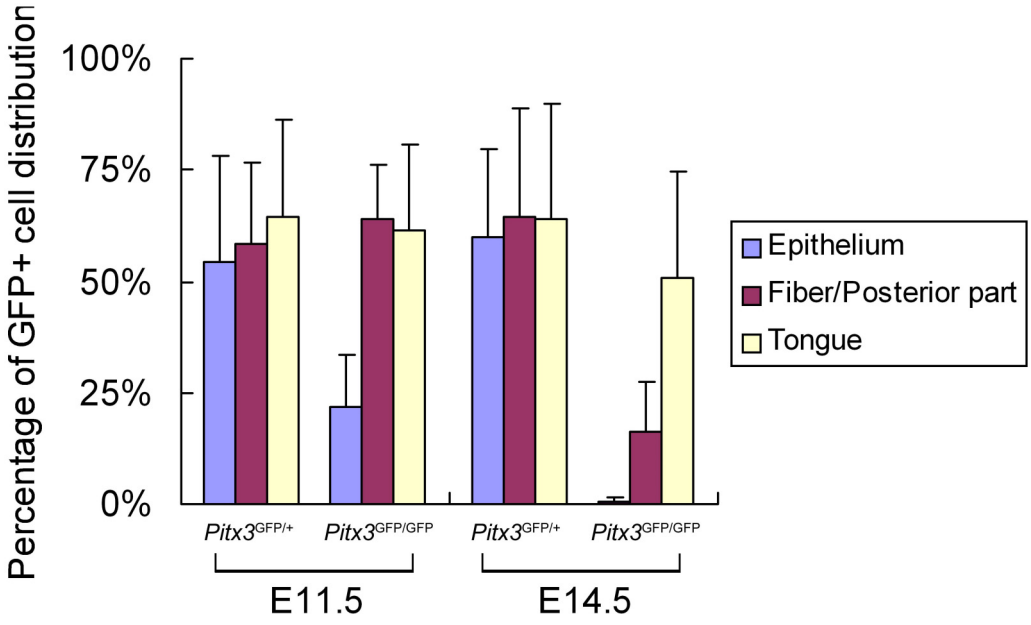

Figure 3-6 Distribution of GFP$^+$ cells in the *Pitx3*$^{GFP/+}$ ↔ wild type and *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras.

In *Pitx3*$^{GFP/+}$ ↔ wild type chimeras, the distribution of *Pitx3*$^{GFP/+}$ cells in the epithelial and fibrous parts were both comparable to the distribution in the tongue (n=7 at E11.5; n=5 at E14.5). In *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras, the distribution of *Pitx3* mutant cells in the lens epithelium was significantly lower than that of the tongue ($p<0.02$ at E11.5, n=7; $p<0.0001$ at E14.5, n=11). There was also underrepresentation of *Pitx3*$^{GFP/GFP}$ cells in the fibrous compartment at E14.5 ($p<0.0001$, n=11).

3.3 Premature fibre cell differentiation of *Pitx3* null lens cells

The lens epithelial cells are predominately cuboidal with strong intercellular adhesion. They are firmly attached to the lens capsule and form an epithelial sheet that covers the anterior surface of the fibre mass. Cell proliferation in the lens is restricted to the epithelial layer. When the progeny of these divisions migrate into the transition zone at the lens equator, they exit from cell cycle, differentiate into post-mitotic fibre cells and persist in the fibre mass throughout life. The balance between proliferation and differentiation is crucial for the maintenance of the lens polarity and the order of cellular architecture that contributes to the transparency and optical properties of the lens (McAvoy et al., 2000).

My chimera experiment has suggested that Pitx3 is autonomously required for the lens epithelial cells. Therefore further experiments were performed to investigate whether the lens epithelial characteristics are changed in *Pitx3* null lenses, by exploiting several key regulators or marker genes normally active in lens epithelial cells. Embryos at E10.5, the lens vesicle stage, were used for most of these experiments because this stage is the last time point when the morphology of *Pitx3* null lens is indistinguishable from that of *Pitx3* wild type or *Pitx3* heterozygous littermates.

3.3.1 Lens epithelial cell identity is not maintained in *Pitx3* deficient mice

Platelet-derived growth factors (PDGFs) are disulfide-bonded homodimers or heterodimers of two polypeptide chains, A and B. Their receptors are two tyrosine kinases, PDGFRα and PDGFRβ, which can also homodimerise or heterodimerise upon binding of the ligand. PDGF-A is expressed by the cells and tissues of the anterior segment of the embryonic eye, including the ciliary body and iridial region.

The receptor PDGFRα is typically expressed at high levels in lens epithelial cells (Morrison-Graham et al., 1992; Reneker and Overbeek, 1996). In this experiment, I used a mouse monoclonal PDGFRα antibody APA5 (Takakura et al., 1997) to examine the lens epithelial identity.

In the E12.5 *Pitx3*$^{GFP/+}$ developing lens, the expression of PDGFRα was restricted to the cytoplasm of the lens epithelium, demonstrating that PDGFRα is a specific lens epithelial marker (Figure 3-7 I-J). At E10.5 and E11.5, PDGFRα was expressed in the entire lens vesicle of the *Pitx3*$^{GFP/+}$ lens (Figure 3-7 A-B, E-F). While no difference in PDGFRα expression was observed between the *Pitx3* null and the *Pitx3* heterozygous control lens at E10.5 (Figure 3-7 A-D), a significant reduction of PDGFRα expression was observed in the *Pitx3* null lens at E11.5 (Figure 3-7 E-H), suggesting downregulation of PDGFRα with the loss of Pitx3.

Cadherins comprise of a large family of calcium-dependent cell adhesion molecules (Gumbiner, 2000). They maintain the structure and functional integrity of epithelial tissues through calcium-dependent interaction at sites of cell-cell contact. During eye development, E-cadherin is expressed ubiquitously in the lens epithelial cells that originate from the surface ectoderm, and is downregulated when these epithelial cells commit to fibre cell differentiation (Xu et al., 2002). In this experiment, the monoclonal antibody ECCD2 was used to detect mouse E-cadherin expression (Shirayoshi et al., 1986; Tada et al., 2005) in the developing lens.

Similar to PDGFRα, E-cadherin was also preferentially expressed in the lens epithelium in E12.5 *Pitx3* heterozygous control lens (Figure 3-7 S-T). At E10.5, the expression pattern of E-cadherin in the *Pitx3* null lens was not different from the

Pitx3 heterozygous control lens (Figure 3-7 K-N). However, the homozygous *Pitx3* mutant lenses exhibited a reduced E-cadherin staining at E11.5 (Figure 3-7 O-R).

These studies together demonstrate that the properties associated with lens epithelial cells could not be properly maintained in *Pitx3* deficient mice. This led to subsequent investigating of the signalling pathway associated with the lens epithelium. Most studies have concentrated on identifying factors that control fibre cell differentiation, and there is strong evidence that members of the FGF growth factor family (Govindarajan and Overbeek, 2001; McAvoy et al., 2000) and the TGFβ superfamily (Belecky-Adams et al., 2002; de Iongh et al., 2001; Faber et al., 2002) are key regulators of this process. In contrast, little is known about the maintenance of the lens epithelium. Recent reports have shown that Wnt signalling and β-catenin are required for lens epithelial cells (Smith et al., 2005; Stump et al., 2003). Wnt signals play important roles in cellular adhesion and differentiation (Hinck et al., 1994a; Hinck et al., 1994b). In the absence of a Wnt signal, a complex containing GSK-3β (glycogen synthase kinase-3β) promotes phosphorylation of β-catenin and its subsequent destruction via ubiquitination and proteolysis (van Noort et al., 2002). In response to a Wnt signal, the β-catenin destruction complex is inactivated and, as a consequence, β-catenin is stabilised. The cytoplasmic accumulation of β-catenin can result in its entry into the nucleus and subsequently binding to members of the TCF/LEF (T cell factor/lymphocyte enhancer binding factor) transcription factor family, and activation of a set of Wnt target genes (Huber et al., 1996). In addition to playing a part in the Wnt signal pathway, the molecule β-catenin can also bind to the calcium-dependent adhesion proteins, cadherins, at the plasma membrane, where it stabilises the cell-cell adhesion complex (Giarre et al., 1998; Hinck et al., 1994a;

Hinck et al., 1994b).

The cessation of Wnt/Fz expression during fibre differentiation is at about the time when the fibre cell-specific marker β-crystallin appears (Ang et al., 2004; Chen et al., 2004). In *LRP6* null mice, Wnt signalling through the β-catenin pathway is blocked and the central lens epithelial layer exhibits irregular shaped β-crystallin positive cells (Stump et al., 2003). These data suggest that Wnt signalling in the anterior epithelium of the lens may have a role in inhibiting aspects of fibre differentiation. Additionally, Wnt signalling has been reported to regulate E-cadherin and Connexin-43 (Ai et al., 2000; Huber et al., 1996; van der Heyden et al., 1998), an adhesion protein which might be regulated by Pitx3 according to the chimeric experiments. On the other hand, the molecule β-catenin is also involved in cell-cell adhesion via cadherin molecules, as conditional deletion of β-catenin in the presumptive lens results in a failure of epithelial cell adhesion, and formation of ectopic cell clusters in the periocular ectoderm expressing β-crystallin and *Prox1*, which is a transcription factor required for fibre cell differentiation (Smith et al., 2005). Thus, the Wnt/β-catenin pathway or β-catenin molecule per se may be involved in the Pitx3-mediated regulation of lens epithelial cell development.

To determine whether there might be a differential expression of β-catenin between lens epithelial cells and fibre cells, I have performed immunohistochemistry using an antibody against β-catenin on E12.5 *Pitx3* heterozygous control lens. Interestingly, the β-catenin expression was restricted to the cytoplasm of the anterior lens epithelium in E12.5 lens and downregulated at the equatorial region where fibre cell differentiation is initiated (Figure 3-8 A-B), indicating that β-catenin may be used as

another lens epithelial marker. However, there is no significant difference between the cytoplasmic β-catenin accumulation of E10.5 *Pitx3* heterozygous control (Figure 3-8 C-D) and *Pitx3* null lens (Figure 3-8 E-F).

3.3.2 *Pitx3* null lens epithelial cells exit cell cycle prematurely

During normal lens fibre cell differentiation, the lens epithelial cells arrest their proliferative status and exit from the cell cycle before their elongation and expression of fibre cell-specific genes. The earlier downregulation of the lens epithelial markers in *Pitx3*$^{GFP/GFP}$ lens led to the examination of the expression of cell cycle inhibitors, which are molecules associated with initiation of lens fibre cell differentiation.

Cell proliferation involves the activation of cyclin-dependent kinase (cdk), which catalyses events required for cell cycle transition. The activity of these cdks is regulated by cdk inhibitors, including p27^{KIP1} (also known as cdkn1b) and p57^{KIP2} (also known as cdkn1c) which inhibit cdks involved in the G1/S transition. Both p27^{KIP1} and p57^{KIP2} are post-mitotic cell markers of which the expression is inversely correlated with cell proliferation. Data from mRNA in situ hybridisation shows that at E11.5, posterior lens vesicle cells begin to express p57^{KIP2} mRNA prior to their elongation and subsequent expression of the lens fibre-specific marker β-crystallin (Lovicu and McAvoy, 1999). Onwards from E11.5, the expression of p57^{KIP2} becomes progressively restricted to cells within the transitional zone.

Mice lacking p27^{KIP1} and p57^{KIP2} show hyperproliferation and impaired differentiation in the developing lens, suggesting that these two cdk inhibitors cooperate to control cell cycle exit and differentiation in the developing lens (Zhang et al., 1997; Zhang et al., 1998). However, the late-stage fibre-cell differentiation

marker γ-crystallin is not affected. Furthermore, *Prox1* mutant lens shows downregulated expression of $p27^{KIP1}$ and $p57^{KIP2}$ but no significant difference with the α- and β-crystallin gene expression (Wigle et al., 1999). These data suggest that the two requirements of fibre cell differentiation, cell cycle exit and crystallin expression, are regulated by separate pathways.

The correlation of $p27^{KIP1}$ and $p57^{KIP2}$ expression with lens epithelial cell cycle withdrawal and the onset of lens fibre differentiation make them useful markers for initiation of lens fibre cell differentiation (Lovicu and McAvoy, 1999).

My immunohistochemistry experiment showed that both $p27^{KIP1}$ (Figure 3-9 E-F) and $p57^{KIP2}$ (data not shown) were only expressed at the most posterior part of the *Pitx3* wild type lens vesicle at E11.5, where fibre cell differentiation is initiated. This is in keeping with the $p57^{KIP2}$ mRNA expression data (Lovicu and McAvoy, 1999). Neither $p27^{KIP1}$ nor $p57^{KIP2}$ was expressed at E10.5 in the wild type lens (Figure 3-9 A-B, I-J). Strikingly, both $p27^{KIP1}$ and $p57^{KIP2}$ were expressed throughout the morphologically normal *Pitx3* null lens vesicles at E10.5 (Figure 3-9 C-D, K-L). They were also highly expressed in cells of the malformed lens structure at E11.5 (Figure 3-9 G-H).

Therefore, *Pitx3* mutant cells show earlier withdrawal from the cell cycle at E10.5 when they still show morphology of epithelial cells of the lens vesicle. These data demonstrate that Pitx3 activity is pivotal for the maintenance of mitotic activity of lens epithelial cells, supporting my chimera result that *Pitx3* null cells do not contribute to the proliferative lens epithelium.

3.3.3 Premature expression of fibre differentiation markers β- and γ- crystallin

Lens cells abundantly synthesise a group of proteins called crystallins. In the mature lens, crystallins are accumulated at high concentrations in a soluble form that maintains the transparency and high refractivity of the lens. Major crystallins are classified into α-, β- and γ-crystallins in mammals. Expression of most of these crystallins is restricted to lens, so their expression is a good indicator of lens differentiation (McAvoy, 1978). Expression of α-crystallin begins at the lens vesicle stage, whereas expression of β- and γ-crystallin usually occurs concomitantly with lens fibre differentiation. During normal lens development, the cell cycle withdrawal of differentiating fibre cells involving the anti-mitotic signals $p27^{KIP1}$ and $p57^{KIP2}$ is regulated co-ordinately with transcriptional activation of β- and γ-crystallins (Lovicu and McAvoy, 1999; Treton et al., 1991). To determine whether the premature cell cycle withdrawal of lens epithelium caused by the loss of Pitx3 is accompanied by fibre cell differentiation, I have investigated the expression of β- and γ-crystallins in the *Pitx3* heterozygous control and *Pitx3* null lenses by staining frozen sections with appropriate antibodies from the lens vesicle stage at E10.5 to the nearly formed lens at E12.5.

At E12.5 in the $Pitx3^{GFP/+}$ lens, β-crystallin was expressed in the fibrous part and γ-crystallin was expressed at the more central part of the fibre cell mass (Figure 3-10 E-F, M-N). Neither staining was observed in the lens epithelial cells in the anterior part of the lenses. Both β- and γ-crystallins were expressed in most, if not all, cells in the collapsed *Pitx3* null lens structure at E12.5 (Figure 3-10 G-H, O-P), demonstrating that some aspects of fibre cell differentiation occur in the $Pitx3^{GFP/GFP}$ cells at E12.5 despite the disorganised structure. Neither β- nor γ-crystallin was

expressed in *Pitx3* heterozygous control lenses at E10.5 when all the cells of the lens vesicle are proliferative epithelial cells (Figure 3-10 A-B). Strikingly, prominent β-crystallin staining covering the entire lens vesicle was observed at E10.5 in the *Pitx3* null lens (Figure 3-10 C-D) when the morphology of the lens vesicle was still comparable to that of *Pitx3* heterozygous control or wild type littermates.

The temporal expression profile of γ-crystallin in the *Pitx3* mutant lens was also changed. Earlier expression of γ-crystallin at E11.5 was found in the malformed *Pitx3* null lens (Figure 3-10 K-L) compared to the *Pitx3* heterozygous control lens (Figure 3-10 I-J).

These data suggest that there is earlier expression of β- and γ-crystallin proteins in the *Pitx3* mutant lens, indicating that fibre cell differentiation starts in lens epithelial cells inappropriately early. These experiments demonstrate that Pitx3 acts as a negative regulator for β- and γ-crystallin expression in the lens epithelium. The detailed mechanism by which Pitx3 regulates crystallin expression, either directly or indirectly, is still unknown.

In summary, the molecular characterisations of *Pitx3* null lens showed precocious downregulation of the lens epithelial markers, PDGFRα and E-cadherin, and premature activation of the cell cycle inhibitors $p27^{KIP1}$ and $p57^{KIP2}$, and the fibre cell-specific proteins β- and γ-crystallin. *Pitx3* null cells therefore show aspects of premature differentiation into fibre cells. This suggests that *Pitx3* is required for the maintenance of lens epithelial cells and to prevent fibre cell differentiation events including cell cycle exit and crystallin expression (Figure 3-11).

3.3.4 Downregulation of the transcription factor *Foxe3* in the *Pitx3* null lens vesicle

The concurrent and distinct functions of numerous transcription factors have been implicated in lens epithelial cell maintenance and fibre cell differentiation (Lang, 2004; Ogino and Yasuda, 2000; Reza and Yasuda, 2004a). The molecular characterisation of *Pitx3* null lens directed attention towards another interesting question, the integration of Pitx3 into the genetic pathway for lens development. I have chosen several transcription factors and examined their expression to determine whether the transcription factor Pitx3 is associated with their regulation during lens development. These candidates included Foxe3, Prox1, Six3 and Pax6.

The winged helix forkhead transcription factor *Foxe3* has a similar expression pattern to that of *Pitx3* in the ocular lens (Blixt et al., 2000; Brownell et al., 2000; Semina et al., 1998; Semina et al., 2000). *Foxe3* is initially expressed in the lens placode at the early stages of mouse lens formation. During differentiation of the lens, expression of *Foxe3* is downregulated in differentiating fibre cells and remains active only in the relatively undifferentiated, proliferative cells of the anterior lens epithelium, thus being a marker of lens epithelial cells (Blixt et al., 2000; Brownell et al., 2000). Functionally Foxe3 promotes proliferation and prevents differentiation in the lens epithelium. It is also essential for lens vesicle closure and subsequent separation of the lens vesicle from the surface ectoderm (Blixt et al., 2000). Loss-of-function of *Foxe3* in mouse mutants *dyl* (*dysgenetic lens*) and *Foxe3* knockout mice give rise to premature differentiation of lens epithelial cells, reduced lens size, and formation of the keratolenticular connection (Blixt et al., 2000; Medina-Martinez et al., 2005). In humans, *FOXE3* mutations cause cataracts and anterior segment mesenchymal dysgenesis (ASMD), similar to those of mutations in

PITX3 (Semina et al., 2001; Semina et al., 1998). The similarity of the expression domain and the mutant phenotypes of lens between *Pitx3* and *Foxe3* prompted an experiment to examine whether Pitx3 regulates Foxe3.

Whole mount mRNA in situ hybridisation was performed 3 times and a total of 8 *Pitx3* wild type and 8 *Pitx3* homozygous mutant embryos were analysed with the *Foxe3* riboprobe. Whilst there was clear expression of *Foxe3* in the forming lens vesicle of E10.5 *Pitx3* wild type embryos (Figure 3-12 A), expression of the *Foxe3* transcript was lost at this stage in the *Pitx3* null lens (Figure 3-12 B), suggesting that the expression of *Foxe3* was regulated by *Pitx3* during lens development. This might partially explain the phenotypes of the *Pitx3* null lens since the *Foxe3* mutant lens also demonstrated diminished *pdgfra* expression and inappropriate expression of $p27^{KIP1}$ and β-crystallin in the lens epithelial cells (Blixt et al., 2000).

The homeobox gene *Prox1* has been shown to be essential for the terminal differentiation of lens fibre cells. Loss of *Prox1* in mice causes abnormal cellular proliferation and downregulation of the cell cycle inhibitors $p27^{KIP1}$ and $p57^{KIP2}$, resulting in a hollow lens due to a failure of lens fibre cell elongation (Wigle et al., 1999). This raised the question of whether the premature cell cycle exit in the *Pitx3* null lens vesicle is caused by deregulation of *Prox1*. To address this question, I examined the expression of *Prox1* by mRNA in situ hybridisation in E10.5 embryos. A total of 7 *Pitx3* wild type and 8 homozygous *Pitx3* mutant embryos were analysed with the *Prox1* riboprobe. In the *Pitx3* wild type lens, *Prox1* was differentially expressed in the posterior part of the lens where fibre cell differentiation is about to take place (Figure 3-12 C). However, *Prox1* expression was extended into the

anterior part of the lens vesicle in *Pitx3* homozygous mutants (Figure 3-12 D). The data indicates that *Prox1* expression is repressed in the lens epithelium by Pitx3 during normal lens development.

The homeobox-containing transcription factor *Six3* in the developing lens is expressed in the lens pit and the lens vesicle, and is later restricted to the anterior lens epithelium. A promoter experiment has suggested that Six3 may be a repressor of the γ-crystallin gene (Lengler et al., 2001). In medaka fish eye development, Six3 antagonises the cell cycle inhibitor geminin to regulate the proliferation of retinal precursors cells, and prevents their premature differentiation (Del Bene et al., 2004). Since Six3 and Pitx3 have similar expression patterns in the developing lens, and both may be negative regulators of the expression of γ-crystallin, mRNA in situ hybridisation was performed to examine whether *Pitx3* regulates *Six3*.

A total of 7 *Pitx3* wild type and 6 homozygous *Pitx3* mutant embryos were analysed with the *Six3* riboprobe. The result showed that the *Six3* transcript was still present in the E10.5 *Pitx3* null lens (Figure 3-12 F), and the expression pattern was not different from that of the *Pitx3* wild type control lens (Figure 3-12 E). This is in keeping with the previous report that *Six3* expression was observed throughout the lens remnants of the *Pitx3* hypomorphic *aphakia* mice (Grimm et al., 1998).

The homeobox gene Pax6 is an essential transcription factor for lens development and functions upstream of most lens regulators including Foxe3, Prox1, Maf and Sox2 (Collinson et al., 2000; Dimanlig et al., 2001; Fujiwara et al., 1994; Lang, 2004; Quinn et al., 1996). In later stages of lens development, Pax6 is expressed exclusively in the lens epithelial cells, and downregulated in the lens fibrous part

(Grindley et al., 1995; Kamachi et al., 1998). A number of promoter studies also propose a later role for Pax6 as a repressor for mouse lens fibre cell-specific *βB1-crystallin* (Cui et al., 2004; Duncan et al., 1998). These data suggest that Pax6 may function to maintain the undifferentiated lens epithelium during later stages of lens development. Thus the loss of *Foxe3* expression or the precocious activation of β-crystallin in the *Pitx3* null lens might be due to a compromised maintenance of Pax6. However, antibody staining in E10.5 and E11.5 embryos showed that Pax6 expression was maintained in both the normal *Pitx3* heterozygous control lens and the *Pitx3* null rudimentary lens (Figure 3-13). A previous report also demonstrates that *Pax6* is expressed in the lens remnants of the *Pitx3* hypomorphic *aphakia* mice (Grimm et al., 1998).

In summary, the *Pitx3* null lens vesicle demonstrated significantly reduced expression of Foxe3 and expanded expression of Prox1. This suggests that Pitx3 regulates Foxe3 in order to maintain lens epithelial properties and normal closure and separation of the lens vesicle, and represses Prox1 directly or indirectly to prevent lens fibre cell differentiation (Figure 3-14).

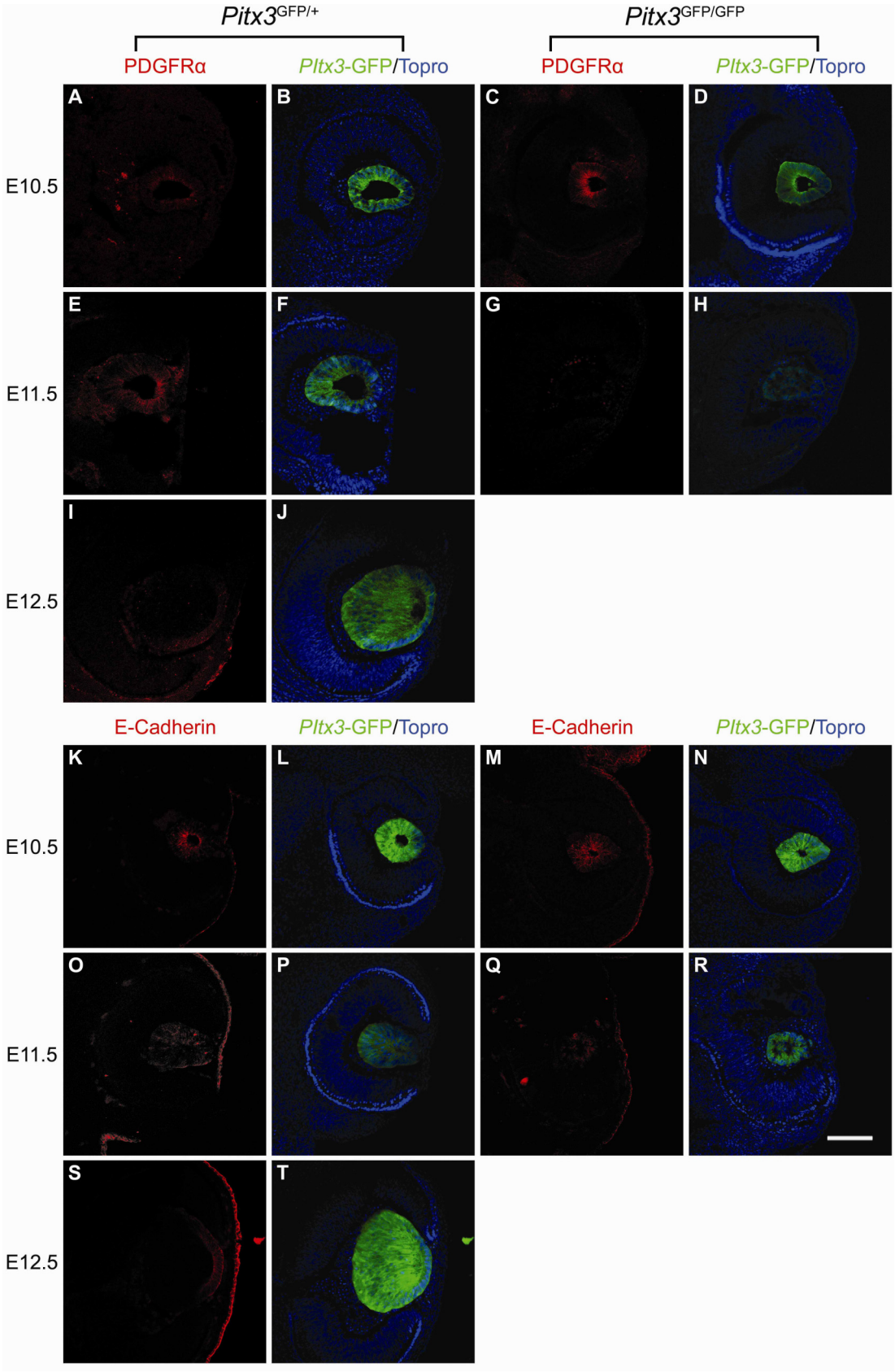
Pitx3GFP/+
Pitx3GFP/GFP
PDGFRα
Pltx3-GFP/Topro
PDGFRα
Pltx3-GFP/Topro
A
B
C
D
E10.5
E
F
G
H
E11.5
I
J
E12.5
E-Cadherin
Pltx3-GFP/Topro
E-Cadherin
Pltx3-GFP/Topro
K
L
M
N
E10.5
O
P
Q
R
E11.5
S
T
E12.5

Figure 3-7 Expression of epithelial markers in *Pitx3* heterozygous null lens

PDGFRα and E-cadherin were expressed in developing lens at E12.5 as epithelial markers (I-J, S-T). They were expressed throughout cells in the lens vesicle stage before differentiation into fibre cells in *Pitx3* heterozygous (A-B, K-L) and homozygous (C-D, M-N) mice. However, the *Pitx3* null lens at E11.5 showed earlier downregulation of PDGFRα (G-H) and E-cadherin (Q-R), compared to the *Pitx3* heterozygous control lens (E-F, O-P).

Scale bar is 100μm.

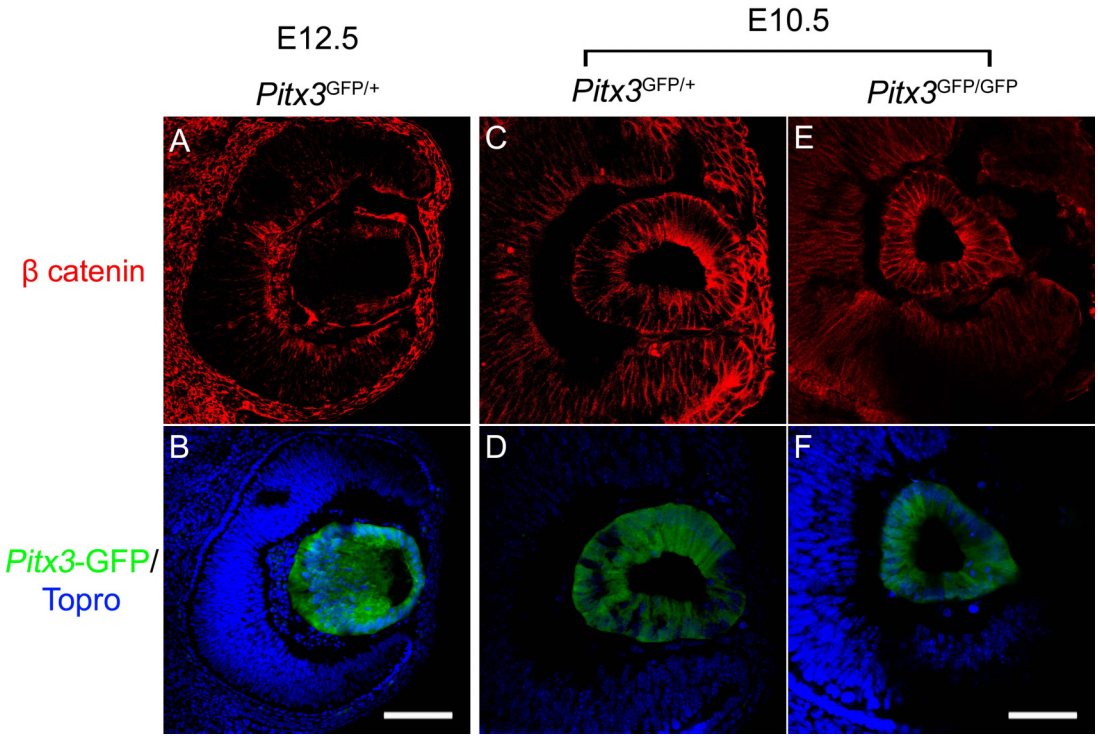

Figure 3-8 β-catenin expression in *Pitx3* heterozygous and null lens

Cytoplasmic accumulation of β-catenin was restricted to the lens epithelial layer in the E12.5 *Pitx3* heterozygous control (A-B), suggesting it might have a functional role in lens epithelial cells. At E10.5, β-catenin was expressed throughout the lens vesicle (C-D). Its expression in the *Pitx3* null lens at E10.5 was not different from the heterozygous control.

Scale bars are 100 μm for A-B, and 50 μm for C-F.

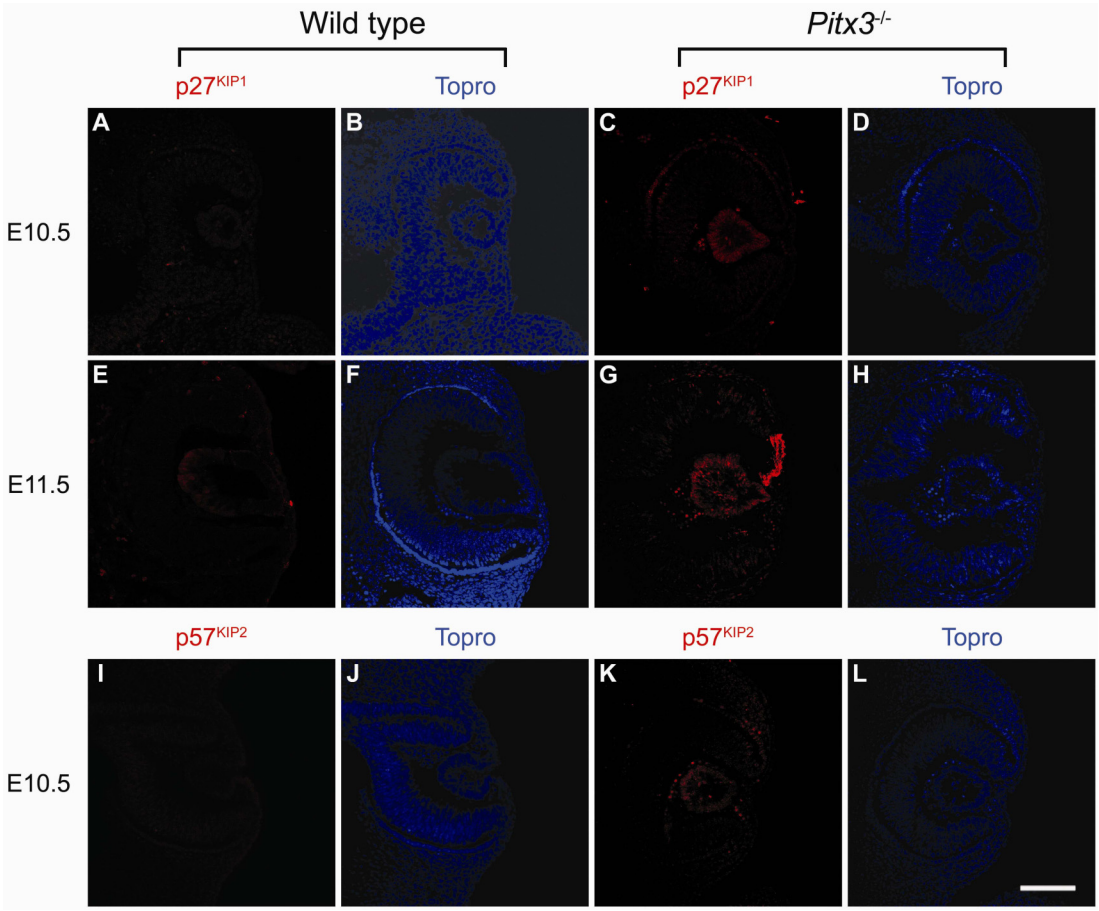

Figure 3-9 Activation of markers for cell cycle exit in *Pitx3* wild type and null lens

Expression of cell cycle exit markers $p27^{KIP1}$ begins at the posterior part of the developing lens when differentiation is initiated (E-F). $p27^{KIP1}$ and $p57^{KIP2}$ were activated in all cells of the lens vesicle in *Pitx3* null mice from E10.5 (C-D, G-H, K-L).

Scale bar is 100 μm.

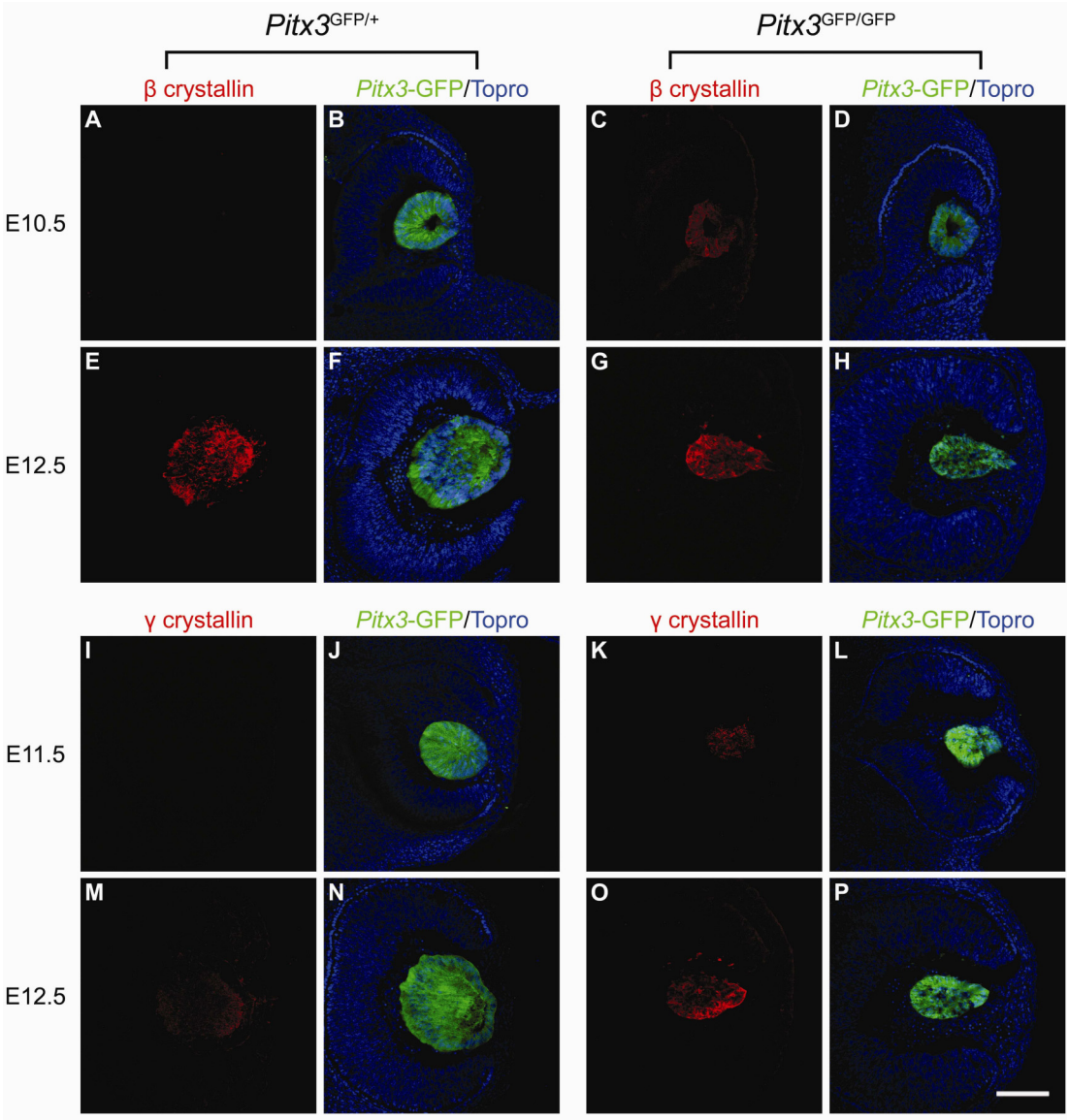

Figure 3-10 Expression of crystallin in *Pitx3* heterozygous and null mice.
β-crystallin was expressed in the fibrous part of the lens (E-F). In *Pitx3* null mice β-crystallin was expressed earlier in the lens vesicle (A-D). γ-crystallin began to be expressed in mature fibre cells from E12.5 (M-N). It was expressed earlier (E11.5) in *Pitx3* null lens (I-L). Expression of both β- and γ-crystallin was maintained in the disorganized E12.5 *Pitx3* null lens (G-H, O-P).
Scale bar is 100 μm.

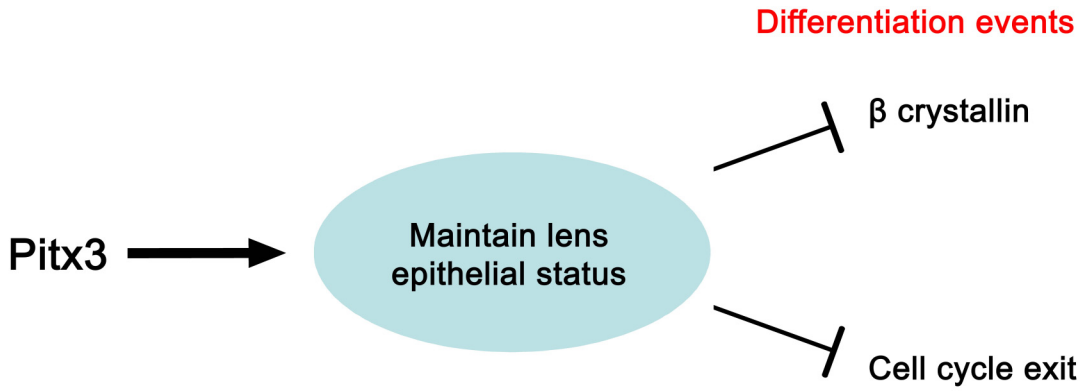

Figure 3-11 Model for the role of Pitx3 in epithelial maintenance in the lens

The *Pitx3* null lens exhibits premature fibre cell differentiation, including earlier downregulation of lens epithelial markers with precocious withdrawal from the cell cycle and expression of the fibre cell-specific protein β-crystallin. This suggests that Pitx3 is required for the maintenance of lens epithelial status.

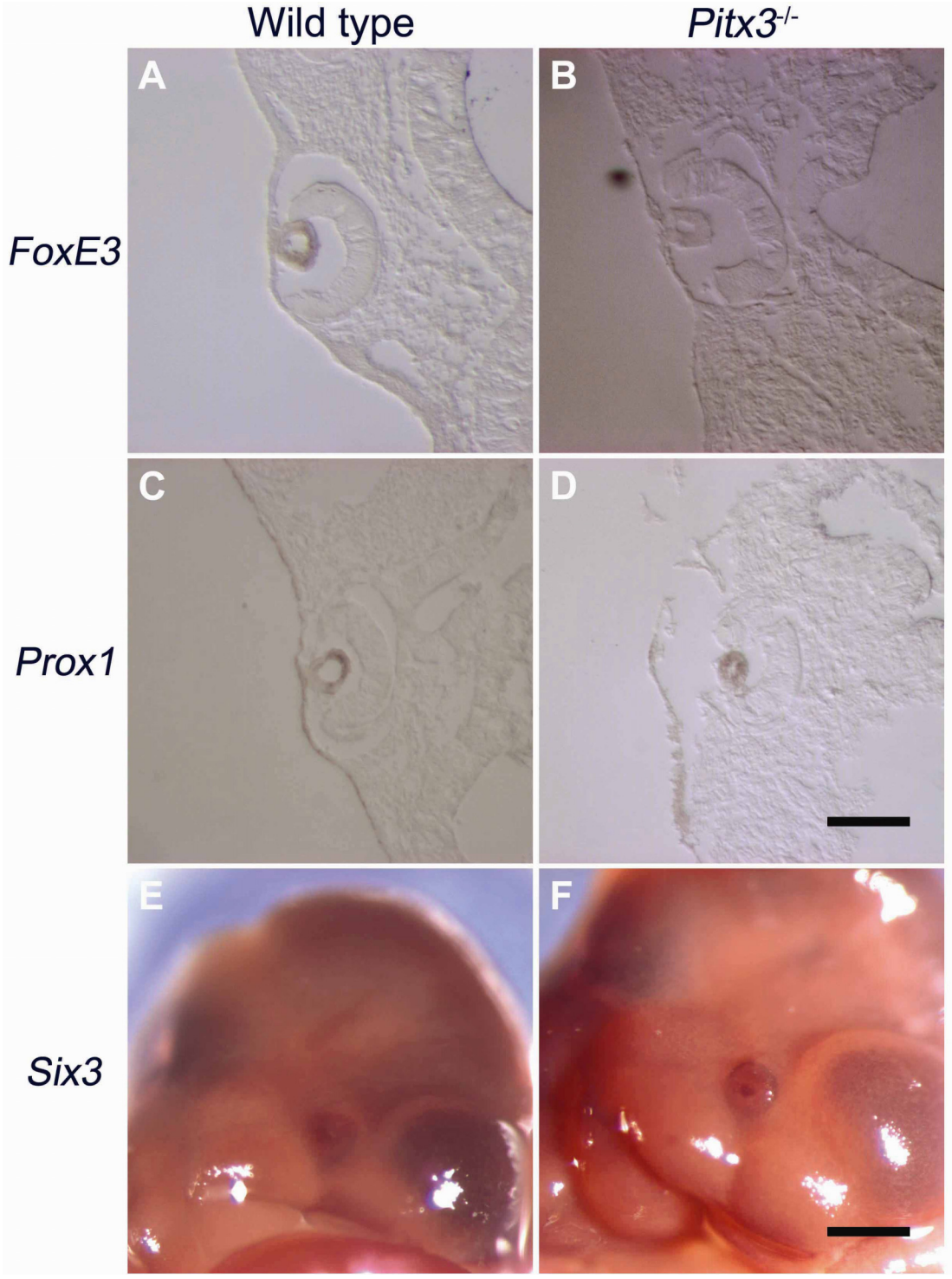

Figure 3-12 Expression of *Foxe3*, *Prox1* and *Six3* in *Pitx3* wild type and null lens vesicle at E10.5

In situ hybridisation showed *Foxe3* expression in the normal lens vesicle at E10.5 but it was downregulated in *Pitx3* null mice (A-B). There was expanded expression of *Prox1* in *Pitx3* null lens vesicle (C-D). The expression of *Six3* was not affected (E-F). Scale bars are 200 μm for A-D, and 2 mm for E-F.

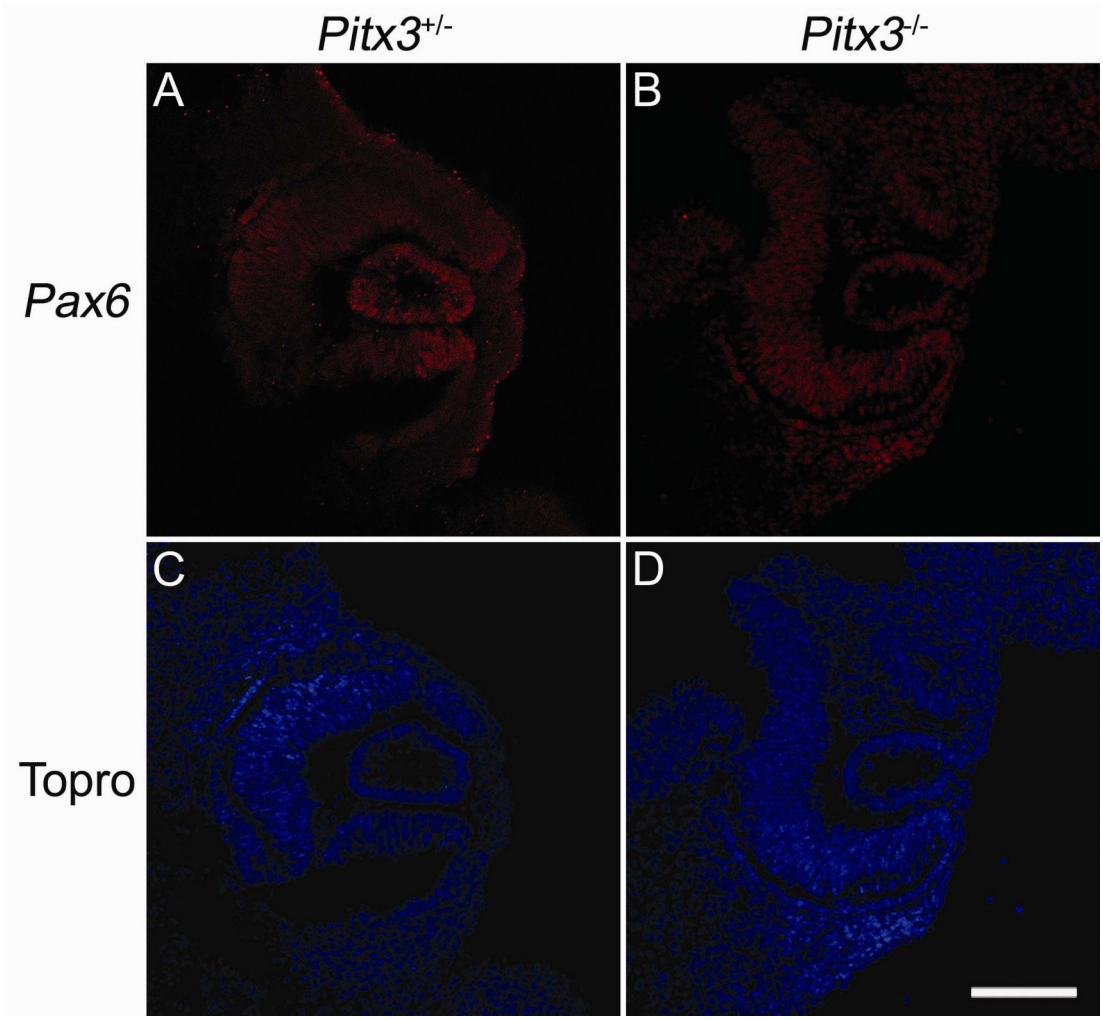

Figure 3-13 Pax6 expression at E10.5 in *Pitx3* heterozygous and null lens

There was no differential expression of Pax6 between heterozygous and homozygous *Pitx3* mutant lenses.

Scale bar is 100μm.

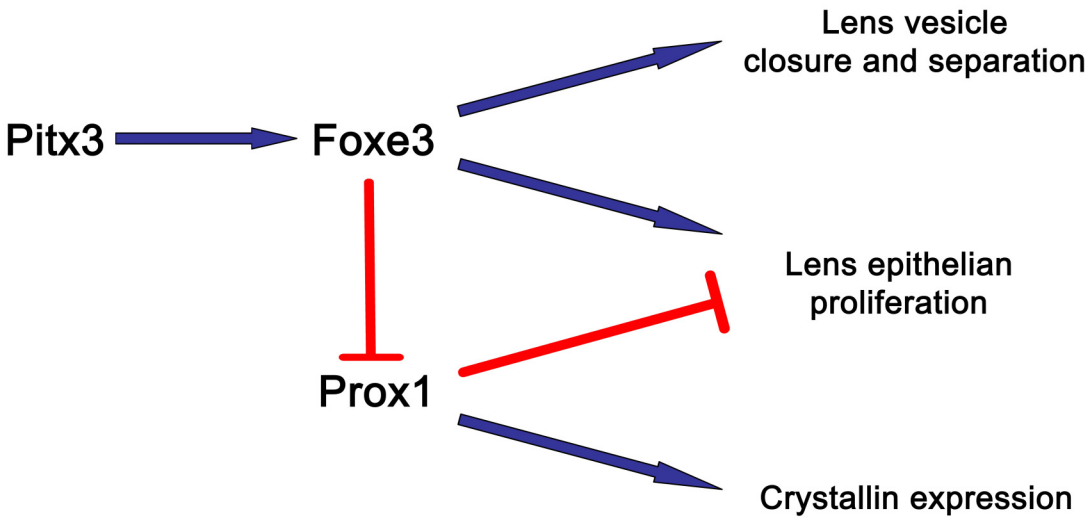

Figure 3-14 Model for Pitx3 regulation of the expression of Foxe3 and Prox1

In the *Pitx3* null lens vesicle, there was downregulation of Foxe3 and expanded expression of Prox1. This suggests that Pitx3 maintains the lens epithelial cell properties partly via Foxe3 regulation, and directly or indirectly represses Prox1 in order to prevent fibre cell differentiation.

3.4 Discussion

3.4.1 The *Pitx3*-GFP reporter for studying lens development

Previously a *Pitx3*-GFP reporter mouse line has been created in our laboratory to analyse Pitx3 expression and to create a mutation in the *Pitx3* gene which results in a *Pitx3* knockout (Maxwell et al., 2005; Zhao et al., 2004). The *Pitx3* hypomorphic *aphakia* studies and the studies of *Pitx3* null mice reveal that the phenotypes of these mice are largely similar (Maxwell et al., 2005), but there is a more severe mDA defect in the *Pitx3* null mice than in *aphakia* mutants. This is probably due to the creation of a true null mutation by gene targeting since the entire *Pitx3* coding sequence is deleted in the targeted *Pitx3* locus (Maxwell et al., 2005; Zhao et al., 2004)(Figure 1-1 C). In contrast, the entire coding sequence remains intact in the *aphakia* mutants and around 5% of the wild type level of *Pitx3* transcript has been detected in E13.5 *aphakia* embryos (Rieger et al., 2001)(Figure 1-1 B). Additionally, in the $Pitx3^{GFP/GFP}$ mice the *Pitx3* coding sequence is replaced with the cDNA of *eGFP*. This knockin-knockout strategy makes the *Pitx3*-GFP reporter mice a useful tool to track the *Pitx3* expressing cells during development. Indeed, previous work carried out in our laboratory have shown that *Pitx3*-GFP expression mirrors that of the Pitx3 RNA and protein during development and in the adult mDA neuron system (Maxwell et al., 2005; Zhao et al., 2004). My study described here confirmed that *Pitx3*-GFP also serves as a reporter for cells of the lens lineage. However, *Pitx3*-GFP was still detectable in differentiated fibre cells in later foetal lens when Pitx3 protein was rarely detected in these cells. This is probably due to the difference in the stability between the Pitx3 and eGFP protein. In the midbrain studies, Pitx3 is expressed in post-mitotic mDA neurons and is maintained into adulthood, so the long

half life of *Pitx3*-GFP does not affect the faithful report of Pitx3 expression. In the developing lens, the Pitx3 protein disappears in differentiated fibre cells, which cannot be correctly reproduced due to the stability and accumulation of eGFP. A solution to this problem as a transcription reporter has been achieved by fusing the degradation domains of other proteins to the C terminus of eGFP to accelerate degradation (Li et al., 1998b). The half life of the destabilised eGFP is only 2 hours, thus such a rapid-turnover fluorescence protein may provide a better temporal transcription reporter to directly correlate gene induction with other cellular and molecular effects.

However, *Pitx3*-GFP is still useful as a lineage marker to track the fate of *Pitx3* expressing cells during lens development in both the *Pitx3* heterozygous and *Pitx3* null mice, at least before E14.5. This strategy has been used in *Ngn2*-GFP heterozygous mice for short-term lineage tracing of the progeny of *Ngn2* expressing precursors because they maintain the GFP expression for a limited time (Andersson et al., 2006a). One example of this use of *Pitx3*-GFP is demonstrated in the current research in the identification of the origin of the persistent lens stalk. The property of the lens stalk has been suggested to be related to surface ectoderm rather than to lens-derived tissue because it expresses Pax6 but not Six3 (Grimm et al., 1998), and it expresses keratin, an epithelial marker for surface ectoderm (Zwaan and Webster, 1985). In contrast, it has also been suggested that the persistent lens stalk may be a result of autonomous deficiency in the lens by a chimera study, in which the lens stalk was not observed when the corneal epithelium derived from surface ectoderm was predominantly mutant (Collinson et al., 2001). Our *Pitx3*-GFP reporter provides direct evidence that the lens stalk corresponds to *Pitx3* expressing lens cells (Figure

3-1 F-G, 3-3 B, 3-4 B). In addition, since *Pitx3* is only expressed in the lens during eye development, the anterior segment dysgenesis in our *Pitx3* mutant mice provides more evidence that correct lens development is essential for the proper development of the anterior chamber of eyes, and anterior segment dysgenesis in *Pitx3* hypomorphic *aphakia* and *Pitx3* null mice arises as a secondary consequence of primary defects in the lens.

3.4.2 Chimera experiments in lens development

Pitx3-GFP is also used as a lineage marker to track the fate of *Pitx3*$^{GFP/+}$ or *Pitx3*$^{GFP/GFP}$ cells in chimera analysis. An important issue of chimera experiments is to distinguish the contribution and genotypes of mutant cells, and provide qualitative, quantitative and spatial information about their distribution in the chimeric tissues. Since I have both the heterozygous and homozygous *Pitx3* mutant ES cell lines, no further molecular tests are needed to distinguish the genotype of the chimeras, which is necessary when embryos from crossmating of heterozygous animals are used for aggregation with wild type embryos. Additionally, in the current chimera studies, *Pitx3*-GFP positive cells could be readily distinguished from wild type cells in the developing lens of chimeras derived from either *Pitx3*$^{GFP/+}$ or *Pitx3*$^{GFP/GFP}$ ES cells. It is straightforward to observe and quantitatively analyse the distribution and morphological change of these mutant cells in the developing lens. Therefore, the production of chimeras with my *Pitx3*-GFP reporter ES cells provides a fine-scale analysis of autonomy of the transcription factor *Pitx3* function at the single cell level. However, the interpretation of this experiment is limited by the fact that the donor ES cells can only be detected by virtue of their *Pitx3*-GFP expression. As there is no constitutive marker for the donor ES cell-derived cells in this system, *Pitx3*

expressing cells which have contributed the lens but have downregulated *Pitx3*-GFP expression are therefore undetectable.

My experiment has extended the finding of the previous chimera report that the gene *aphakia* acts cell autonomously in the lens (Liegeois et al., 1996). Chimeria experiments using *aphakia* mutants have also been used in the blastocyst complementation assay for developing lenses because mutant cells are genetically impaired to form a lens autonomously and the lens will derive entirely from donor cells (Liegeois et al., 1996; Zhao et al., 2006). Our highly sensitive eGFP reporter demonstrated that a certain proportion of lens cells are still *Pitx3*$^{GFP/GFP}$ derived in the phenotypically rescued lens. Therefore using *aphakia* blastocysts in this lens complementation system may have problems for genetic analysis of some candidate genes of lens development and the interpretation should be very careful.

In the homozygous chimeric embryos in which relatively normal lens development was maintained by the wild type cells, the distribution of *Pitx3* null cells was significantly lower in the lens epithelium, compared to that of heterozygous chimeras (E/T ratio 0.41 ± 0.23 vs. 0.84 ± 0.31, $p<0.02$), at E11.5. The *Pitx3*$^{GFP/GFP}$ cells were more severely underrepresented in the lens epithelium at E14.5 (E/T ratio 0.0057 ± 0.0134 vs. 0.93 ± 0.12, $p<0.0001$). Thus, there is an autonomous requirement for functional *Pitx3* for normal development of lens epithelial cells.

Quantitative analysis did not show underrepresentation of *Pitx3* mutant cells in the fibrous part at E11.5 although qualitative observation revealed segregation of some *Pitx3*$^{GFP/GFP}$cells. By E14.5, *Pitx3* mutant cells were underrepresented in the lens fibres as well as the lens epithelium in *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras (F/T ratio

0.34 ± 0.22 vs. 0.97 ± 0.04, $p<0.0001$). The lens epithelium acts as the source of lens fibres so this underrepresentation in E14.5 lens fibres (Figure 3-5 D) may partly reflect the earlier depletion of *Pitx3* null cells from the lens epithelium (Figure 3-5 A).

An interesting finding in my *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras is the non-random distribution into separate populations and physically excluded buddings of *Pitx3* mutant cells at the fibrous compartment, especially in E14.5 chimeric lenses. Rare *Pitx3* null cells differentiated into elongated fibre cells and integrated well in the fibrous compartment. These events are most likely the result of different adhesive properties of wild type and *Pitx3* mutant cells at this stage in *Pitx3*$^{GFP/GFP}$ ↔ wild type chimeras. The transcription factor *Pax6* has also been suggested to be involved in the regulation of surface/adhesion molecules because of similar patterns of segregation or physical exclusion observed in chimera studies (Collinson et al., 2000; Collinson et al., 2001; Collinson et al., 2003; Quinn et al., 1996). The sharp partitioning of the *Pitx3*$^{GFP/GFP}$ cells and wild type cells suggests a mechanism operating at an earlier developmental stage, most likely during differentiation that sorts the cells into large patches.

Downregulation of Pitx3 expression in the differentiated lens cells suggests *Pitx3* may not be functionally activated in fibre cells. The abnormality of surface/adhesion molecules in the mutant fibre cells may be caused by deficiencies in the mutant lens epithelium, direct effects on the differentiating fibre cells, or both. Another explanation of the segregation picture is that asynchronous development, i.e. premature or impaired differentiation, of *Pitx3* mutant cells makes their expression of

surface/adhesion molecules different from wild type cells. This question needs to be further investigated.

In addition to the application in lens development research, chimeras derived from *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ ES cells have also been used to study the autonomy of Pitx3 in mDA development by other members in our laboratory. In heterozygous chimeric mice, the *Pitx3*-GFP^{+} cells contributed to both the VTA and the SN area. On the other hand, in homozygous chimeras the *Pitx3*-GFP^{+} cells showed contribution to the VTA but rarely to the SN. The ratio of the number of *Pitx3*-GFP^{+} cells in the SN to the number of *Pitx3*-GFP^{+} cells in the VTA was compared between chimeras. The VTA acts as a control region where no variation is expected between *Pitx3* heterozygous and the *Pitx3* null ES cell contribution as the VTA phenotype is the same in *Pitx3* heterozygous and *Pitx3* null E14.5 embryos. It has been demonstrated quantitatively that *Pitx3* null cells were impaired in contributing to the SN, as the ratio of SN:VTA *Pitx3*-GFP^{+} cells in homozygous chimeras was much lower than in heterozygous chimeras (Maxwell, 2006). Therefore, these chimera experiments have shown a cell-autonomous requirement of Pitx3 for both SN mDA neurons and lens epithelium during development.

3.4.3 Premature differentiation of lens cells in the absence of Pitx3

Further immunohistochemistry studies in this chapter have revealed a reduced expression of the lens epithelial markers PDGFRα and E-cadherin, showing that *Pitx3* null cells lose their epithelial properties. Additionally, *Pitx3* null lens prematurely activated fibre cell-specific β- and γ-crystallins. Furthermore, cells throughout the *Pitx3* null lens vesicle expressed cell cycle inhibitors p27^{KIP1} and

$p57^{KIP2}$, suggesting that these *Pitx3* null cells has arrested the cell cycle. This supported the chimera data that *Pitx3* null cells hardly contribute to the proliferative lens epithelium.

It has been shown that PDGF-A is a potent mitogen for lens epithelial cells (Kok et al., 2002), and signalling through PDGFRα is believed to mediate the growth of lens epithelium (Brewitt and Clark, 1988; Reneker and Overbeek, 1996). Therefore, it may be argued that PDGFRα also has a functional role for lens epithelial maintenance in addition to being a lens epithelial cell-specific marker. It has been reported that mice of the *patch* mutation, which includes a deletion of the PDGFRα gene (Stephenson et al., 1991), display abnormally shaped lenses with a decreased number of fibre cells (Morrison-Graham et al., 1992). More recent targeted mutation experiments have shown mice that lack the PDGFRα have apparently normal lenses, and the *patch* phenotype is multigenic with some but not all features attributed to the loss of the PDGFRα (Soriano, 1997). The lack of a phenotype may indicate that PDGFRα receptor pathway is not essential or may be functionally compensated in maintaining lens epithelial proliferation in vivo. The diminishment of PDGFRα indicates that *Pitx3* null cells lose lens epithelial characteristics, rather than mutant epithelial cells experience a decreased growth factor receptor signalling.

I have also examined the expression of β-catenin, which is a dual-functional molecule with a critical role in both Wnt signal transduction and cell-cell adhesion (Giarre et al., 1998). In the immunohistochemistry of E12.5 $Pitx3^{GFP/+}$ lens, β-catenin was expressed exclusively in lens epithelial cells and disappeared in the lens fibre cells, implying a functional role for lens epithelial cell adhesion. This also indicates

that β-catenin may be another lens epithelial marker during lens development. Further immunohistochemistry data has shown that the β-catenin expression pattern was not altered in the E10.5 *Pitx3* null lens vesicle, compared to the *Pitx3* heterozygous controls. This reveals that β-catenin-mediated epithelial cell adhesion is not altered in *Pitx3* null lens at least at E10.5. However, it is still an unsolved question whether the Wnt signalling pathway is involved or not. It has been reported that Wnt signalling is essential for the maintenance of lens epithelial cells and to prevent fibre cell differentiation (Stump et al., 2003). However, my antibody staining pattern of β-catenin was mostly cytoplasmic rather than nuclear localisation. Although cytoplasmic accumulation of β-catenin is a common result of Wnt activation (Giarre et al., 1998), nuclear localisation is believed to provide more direct evidence of the activation of Wnt signalling to its target genes (Cong et al., 2003). It has been suggested that the antibody may be used against non-phosphorylated β-catenin to confirm the activation of the Wnt/β-catenin signalling pathway. However, it is worth noting that most of the reactivity of non-phosphorylated β-catenin in the lens epithelium is still cytoplasmic and only punctate reactivity is detectable in some nuclei (Stump et al., 2003). Therefore, alternative strategies should be used to investigate the role of the Wnt/β-catenin pathway in Pitx3 regulation of lens development. For example, lens epithelial cells can be placed in culture and used to determine the ability of purified growth factors to regulate lens epithelial cell proliferation or fibre cell differentiation (Chamberlain and McAvoy, 1989). In this primary cell culture system the Wnt/β-catenin pathway may be activated by treatment with lithium, which stabilises β-catenin by inhibiting GSK-3β (Hedgepeth et al., 1997; Klein and Melton, 1996), and the behaviour of *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ lens cells can be compared.

Fibre cell elongation, which is a morphological hallmark of lens fibre cell differentiation, is severely impaired in both *Pitx3* hypomorphic *aphakia* and the *Pitx3* null mice. Therefore it has been suggested that loss of Pitx3 leads to impaired lens differentiation. More detailed molecular studies in lens development indicate that the lens fibre cell differentiation process can be uncoupled into several events, including cell cycle arrest, crystallin accumulation and cell elongation, among which each event can be regulated by different molecular pathways. Fibre cell elongation is a complex morphological change which involves precise regulation of cytoskeleton and cell-cell adhesion properties (Piatigorsky et al., 1972; Xia et al., 2006), appropriate membrane permeability, intracellular osmotic pressure and concentration of the structural proteins including crystallins (Bassnett et al., 1994; Beebe et al., 1979; Parmelee and Beebe, 1988; Wang et al., 2005; Zelenka, 2004). Therefore, the impairment of elongation in *Pitx3* null lens might be a secondary consequence of abnormal regulation of crystallins or surface/adhesion molecules.

FGF signalling could be a candidate pathway involved in Pitx3 regulated lens development. In vivo studies using transgenic mice have demonstrated that lens-specific expression of several different FGF ligands induce lens epithelial cells to exit from the cell cycle prematurely and undergo ectopic fibre cell differentiation (Robinson et al., 1995). Experiments using the rat lens epithelial explant culture system suggested that a high concentration of FGF induces fibre cell differentiation events, including β- and γ-crystallin accumulation and cell elongation. The ERK pathway is responsible for FGF-induced fibre cell elongation and expression of the fibre cell-specific protein filensin (Lovicu and McAvoy, 2001). Phosphorylated ERKs are expressed in the rat lens epithelial layers and the transition zone, similar to

the expression domain of Pitx3. However, FGF-induced β-crystallin accumulation in lens cell culture experiments is independent of ERK signalling, suggesting that an as yet unidentified pathway is involved. Further detailed investigation about the functional roles of FGF signalling in Pitx3 regulated pathway could be achieved by primary culture of *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ lens cells with the treatment of FGF and its inhibitors to examine the proliferation, cell elongation and expression of lens fibre cell-specific proteins.

In summary, *Pitx3* null lens cells show precocious activation of fibre cell differentiation programmes, suggesting that Pitx3 maintains the lens epithelial cell property (Figure 3-11).

3.4.4 Comparison of Pitx3 to other transcription factors involved in lens epithelium development

Transcription factors regulating lens development can be classified into two groups: those involved in lens fibre cell differentiation and those that maintain the lens epithelium. Known transcription factors functionally required for lens fibre cell differentiation include Prox1, c-Maf and Sox1. Comparison of the phenotype of *Prox1*, *c-Maf* and *Sox1* knockout mice suggests distinct but overlapping functions for these genes in fibre cell differentiation. All three mutants show impairment in fibre cell elongation to fill the lens vesicle, so they all have a similar lens malformation characterised by a hollow lens (Kamachi et al., 1998; Kawauchi et al., 1999; Ring et al., 2000; Wigle et al., 1999).

Transcription factors required for lens epithelial cells include Foxe3 and Pitx3. My experiments have revealed that Pitx3 is a more potent transcription regulator than

Foxe3 in the maintenance of lens epithelial cells (Chapter 3.3). There are a lot of similarities in the expression domain and the null phenotypes between these two transcription factors (Blixt et al., 2000; Brownell et al., 2000; Semina et al., 2001). Both mutants have the impaired growth of the lens structure which subsequently makes abnormal folding of the retina tissue and shrinkage of the eye, thus the mutant mice can not open their eyes. The lens vesicle of both mutants is not detached from the surface ectoderm and the persistent lens stalk interferes with the formation of the anterior chambers of the eye. The *Foxe3* mutant mice show inappropriate expression of β-crystallin in some lens epithelial cells whereas the *Pitx3* mutant mice show precocious expression of β-crystallin protein throughout the lens vesicle. Both mutants show earlier expression of $p27^{KIP1}$ and expanded expression of Prox1, which are involved in cell cycle arrest and initiation of differentiation. The *Foxe3* null lens shows diminished expression of the lens epithelial marker PDGFRα in the small population of lens epithelial cells that remain; however, the lens epithelial cells in the *Pitx3* null mice completely disappear and the whole lentoid structure is absorbed before birth. Therefore, the phenotype of *Pitx3* hypomorphic *aphakia* or *Pitx3* null mice is much more severe than that of *dyl* mutants or *Foxe3* knockout, supporting that Foxe3 is a Pitx3 downstream target. This is in agreement with a recent report demonstrating that *pitx3* is required for *foxe3* expression in zebrafish lens development (Shi et al., 2006b). Since mutation of *Foxe3* also reveals an expanded *Prox1* expression pattern in the lens epithelium, suggesting that *Prox1* is genetically repressed by Foxe3 in a mouse lens developmental pathway (Blixt et al., 2000), further investigation is needed to examine whether Pitx3 represses the expression of *Prox1* directly or via the *Foxe3* pathway.

Pax6 and Six3 have also been implicated in a similar function. They are both evolutionarily conserved homeodomain transcription factors, and share a temporally and spatially overlapping expression pattern in the developing eye, including the optic stalk, optic vesicle, lens pit, lens vesicle, and later exclusively in lens epithelial cells and downregulated in differentiated lens fibre cells. Mutual regulation of these two transcription factors in the developing lens has been suggested previously (Goudreau et al., 2002). Although both Pax6 and Six3 function in broader regions at earlier stages, they may be involved in lens epithelial cell maintenance at later stages of lens development. It has been shown that lens-specific expression of Six3 or Pax6 in transgenic mice gives rise to upregulation of PDGFRα and defective maturation of lens fibre cells (Duncan et al., 2004; Goudreau et al., 2002). Ectopic expression of Pax6 in the lens fibre cells reduces the transcript levels of *βB1-crystallin* and the protein levels of c-Maf, which is an important transcription factor in fibre cell differentiation, implying that the loss of Pax6 expression is essential for fibre cell differentiation and maturation (Duncan et al., 2004). *Pax6* haploinsufficient mice and mice in which an upstream ectoderm enhancer of *Pax6* has been deleted ($Pax6^{\Delta EE/\Delta EE}$) both show defects in lens epithelial proliferation, smaller lens and a persistent lens stalk (Dimanlig et al., 2001; Hill et al., 1991). Promoter experiments have also shown that Pax6 and Six3 may be repressors of β- and γ-crystallin genes, respectively (Cui et al., 2004; Duncan et al., 1998; Lengler et al., 2001). Considering their inversely related temporospatial expression pattern to crystallin genes during lens development, it has been suggested that Pax6 and Six3 may be inhibitors of crystallin gene expression in lens epithelial cells while the activators such as Prox1 and c-Maf are expressed. During fibre cell differentiation the repressors Pax6 and Six3 disappear and crystallin expression begins in the fibre cells.

Since the expression of Pax6 and Six3 is restricted to the lens epithelial cells, their remaining expression throughout the *aphakia* lens remnants has led to the hypothesis that the *aphakia* eye lacks fibre cell differentiation (Grimm et al., 1998). However, in my experiments the expression of β- and γ-crystallin observed in the *Pitx3* mutant lens provides direct evidence that *Pitx3* null cells activate aspects of fibre cell differentiation. The remaining expression of Pax6 and Six3 in the β- and γ-crystallin expressing *Pitx3* null lens remnants implicated that the cooperation of Pitx3 might be required for the repression effect of Pax6 and Six3 on the crystallin genes. A good example is that indirect recruitment of Prox1 via other molecules collaborates with c-Maf in order to activate the mouse *βB-crystallin* promoter (Chen et al., 2002). A more detailed β- or γ-crystallin promoter experiment may be helpful to examine the interaction of Pitx3 with the repressors Pax6 and Six3, and the activators c-Maf and Prox1 in lens cells.

Microarray and RT-PCR analysis of *Pax6* heterozygous lens has suggested that Pitx3 may be a direct target of Pax6 (Chauhan et al., 2002a; Chauhan et al., 2002b). The overlap in phenotypes of *Pitx3* null, *Foxe3* null and *Pax6* haploinsufficient mice, and the similarity of clinical manifestation of ASMD with cataracts from heterozygous mutations in human *PAX6*, *PITX3* and *FOXE3* suggests that they may be part of a pathway involved in lens development. These data lead to the hypothesis that the transcription factor Pitx3 is located downstream of Pax6 and upstream of Foxe3 in the genetic regulation of lens development. Whether Pitx3 expression is directly regulated by Pax6 in the lens remains to be further investigated.

The transcription factor Six3 is also expressed at broader regions at earlier stages before being progressively restricted to lens epithelium, thus making it possible to function upstream of Pitx3. Further research is needed to investigate whether Six3 regulates or cooperates with Pitx3 during lens development.

3.4.5 Functional homology of Pitx3 in vertebrates

Zebrafish *pitx3* and *Xenopus XPitx3* are more closely related to each other than to their mammalian Pitx3 orthologs. The *Xenopus Xpitx3* and zebrafish *pitx3* are both expressed in the prospective lens and pituitary (Dutta et al., 2005; Pommereit et al., 2001). They may have subsumed the functions of mouse *Pitx1* and *Pitx3*. Mouse *Pitx1* expression is required for development of Rathke's pouch and subsequently all endocrine adenohypophyseal cells (Gage et al., 1999; Lanctot et al., 1999; Szeto et al., 1999). However, mouse pituitary does not express *Pitx3*, and *Pitx3* hypomorphic *aphakia* mice do not have any apparent pituitary defect (Gage et al., 1999; Semina et al., 1998; Smidt et al., 1997). Additionally, human foetal mesencephalon, but not anterior pituitary, expresses PITX3 (Pellegrini-Bouiller et al., 1999).

In zebrafish, the *pitx3* expressing cells constitute an equivalent domain of cells that can form either pituitary or lens, and in which a non-hedgehog signal initially specifies the placode field (Dutta et al., 2005). The *smoothened* mutant embryos that cannot transduce hedgehog signals lack pituitary and instead form ectopic lenses. The cell fate depends on the level of hedgehog signalling: the median *pitx3* expressing cells respond to hedgehog signalling and acquire pituitary characteristics, whereas in the lateral region absence of hedgehog signal might favour lens specification (Dutta et al., 2005). At later stages, *pitx3* expression in the eye is restricted to the zebrafish lens

equator (Shi et al., 2005). In the zebrafish *pitx3* knockdown experiment, the morphant lenses display disorganised and irregularly shaped cells, similar to the collapsed lens vesicle in *Pitx3* null mice. Furthermore, immunohistochemistry has shown that the morphologically disordered morphant lens epithelial cells lack the molecular characteristics of the wild type lens epithelial cells. This supports a conserved requirement of Pitx3 for normal lens development. However, the *pitx3* morphants also exhibit retina defects that are characterised by cell death and a significant reduction in some retinal cell types. The retina defects highlight a difference to the mouse *Pitx3* null phenotype, and are likely to be a non-autonomous effect from the aberrant lens development (Shi et al., 2005).

Xenopus Xpitx3 transcripts are found in the thickening lens placode during early steps of eye cup function. In the later stages, expression of *Xpitx3* in the lens is restricted to the anterior epithelial layer but is not detected in lens fibres, similar to the mouse *Pitx3* expression pattern (Pommereit et al., 2001). Overexpression and inhibition of the *Xpitx3* function early in development alters the size of the medial pre-optic field as assessed by the markers *Pax6* and *Six3* (Khosrowshahian et al., 2005). *Xpitx3* knockdown results in abrogation of lens function, and in the absence of this critical cue, further prohibits induction of the retina, a phenotype similar to that of zebrafish. Therefore, although zebrafish *pitx3* and *Xenopus XPitx3* are also expressed in prospective pituitary, there is a conserved requirement of Pitx3/Xpitx3/pitx3 for the lens epithelial cells during development.

3.4.6 PITX3 and human cataracts

Mutation of *PITX3* in humans is responsible for autosomal dominant posterior polar cataract, which is a clinically distinctive opacity located at the back of the lens and can have a marked effect on visual acuity. The human PITX3 gene is mapped to 10q25 (Semina et al., 1998). The genomic structure of PITX3 appears to be the same as that of the mouse gene. Human and mouse PITX3/Pitx3 are 88% identical at the nucleotide level throughout the coding region and 80% identical in the 3' UTR. At the protein level, PITX3 and Pitx3 appear to be 99% identical (4 amino acid substitutions out of 302), with 100% identical in the homeodomain.

Detailed sequence analysis has revealed that a recurrent 17 base pair duplication (657ins17bp) in PITX3 is primarily associated with this familial cataract (Addison et al., 2005; Berry et al., 2004; Semina et al., 1998). The duplication in exon 4 results in a frameshift in codon 220 and produces an aberrant protein consisting of 94 additional residues. In addition to this duplication mutation, another recently identified mutation in PITX3 is 650delG (Berry et al., 2004; Bidinost et al., 2006). This novel locus in 10q25 encompassing PITX3 for the posterior polar cataract has been termed CPP4 (Berry et al., 2004). Two cases that are homozygous for this *PITX3* mutation have been reported to display microphthalmia and significant neurological impairment (Bidinost et al., 2006). It has been suggested that the mutation CPP4, which is located outside the homeodomain region, alters the complex protein-protein interaction which imparts the specificity and efficiency to homeoprotein function (Semina et al., 1998), but further investigation is needed for the mechanisms giving rise to the specific manifestation of the ocular disease.

Other mutation of known genes associated with posterior polar cataract include *CRYAB* (Berry et al., 2001). Since my mouse experiments have revealed that Pitx3 is involved in the regulation of crystallin expression, it would be interesting to investigate whether the *PITX3* mutation in humans is associated with crystallin regulation.

Although the posterior polar cataract is the predominant feature of the *PITX3* mutation, some affected families have ASMD in addition to the cataract. Within these families the phenotype is variable with only some affected members of the pedigrees displaying ASMD (Burdon et al., 2006). This variability within families and between families suggests the presence of modifying genes or environmental factors for ASMD. This is also in keeping with my *Pitx3* null mouse experiments that the lens deformity is the predominant phenotype and the dysgenesis of the anterior chamber is secondary to the developmental defect of the lens.

A number of mutations in transcription factors have been shown to cause ASMD and cataracts, including PAX6, FOXE3, MAF and EYA1. In my mouse experiments, expression of *Foxe3* is regulated by Pitx3. Other studies have suggested that Pitx3 may be regulated by Pax6 in lens development (Chauhan et al., 2002a; Chauhan et al., 2002b). A notable overlap in the phenotypes arising from mutations in PAX6/Pax6, PITX3/Pitx3 and FOXE3/Foxe3 suggests that they may be part of a pathway that involves mutual activation during human lens development (Semina et al., 2001; Semina et al., 2000).

Chapter 4

Developing the *Pitx3*-GFP ES cell systems for the identification of midbrain dopaminergic regulators

4.1 Introduction

4.1.1 A lineage-specific reporter ES cell system

A key property of ES cells is that their genome can be manipulated without compromising their pluripotency. Therefore these cells provide an excellent tool to study the cellular and molecular basis of commitment and differentiation events during early development. To achieve this goal, the commitment or differentiation events should be induced reproducibly in a controlled, stepwise fashion. It should be possible to identify differentiated cells at various stages along the lineage and these cells should express expected patterns of markers and exhibit functional capacity during stages of development. Since current differentiation protocols generally result in a mixed population of cells which are enriched only to a limited degree for the cells of interest, ES cells with an engineered lineage marker can provide a useful tool for evaluating molecules which regulate cell fate and/or differentiation (Aubert et al., 2002; Li et al., 1998a; Ying et al., 2003b). In order to study mDA fate specification, we have generated ES cell lines in which a reporter has been linked to reproduce the expression of Pitx3.

Reporter proteins can generally be visualised due to bioluminescence, fluorescence or enzymatic properties. The gene encoding the reporter protein may be expressed under the control of a specific promoter by a number of methods. For example, in a traditional transgenic approach, a construct can be made containing a cell type-specific promoter fused to a reporter gene, which is introduced into ES cells. However, the integration of the reporter gene into the genome is random and the neighbouring sequences may interfere with the regulation of the reporter gene

expression, thus obscuring reporter gene analysis. To avoid this problem, the reporter gene may be placed under an endogenous gene promoter via homologous recombination in ES cells, thus it can recapitulate the expression of the gene of interest.

A number of proteins have been successfully used as reporters, including a vital fluorescent reporter such as green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoris* (Chalfie et al., 1994). Several mutants of wild type GFP have been created to allow increased brightness and thermostability in mammalian expression. For example, the enhanced GFP (eGFP), the most popular variant for use in mice, has two amino acid changes in the vicinity of the fluorophore (F64L and S65T), thus it is more thermostable at 37ºC and it is 350-fold brighter than wild type GFP (Hadjantonakis et al., 2003). Whilst eGFP may not be as sensitive as some enzymatic reporters it has the advantage that no substrate or reaction cofactors are required. In addition, eGFP can be directly visualised under the fluorescence microscope in both fixed tissue and live cells. This non-invasive visualisation of GFP opens up the possibility for direct observation and purification of these cells by fluorescence-activated cell sorting (FACS) for further experiments without fixing the cells (Aubert et al., 2003; Maxwell et al., 2005; Wang et al., 1998).

Other options for reporters include the enzymes β-galactosidase (β-gal), luciferase and β-lactamase. Enzymatic reporters are sensitive detectors of gene expression as each molecule of enzyme can catalyse many reactions to produce the detected product. However, a substrate must be present in order to facilitate the visualisation of the reporter expression and it can be difficult to deliver the substrate to live cells.

A good example of the application of enzyme reporter is the improvement of subcellular resolution by the axon-targeted fusion protein tauLacZ. This recombinant protein fuses β-gal, the product of the *E.Coli LacZ* gene, to bovine tau, a member of the microtubule-associated protein family (Callahan and Thomas, 1994; Callahan et al., 1998). This reporter labels cell bodies and axons which are sensitively detected by X-gal staining. The *Pitx3*-tauLacZ reporter system is being developed by other people in our laboratory for in vivo analysis of mDA axon pathfinding and in vitro experiments for testing important chemoattractive or chemorepulsive factors for axon guidance during mDA development (Vives and Li, in preparation).

The drawback of either the GFP or β-gal reporter is the stability and accumulation of these reporter proteins in the cell when the gene of interest is no longer expressed. Since Pitx3 is expressed and maintained in post-mitotic mDA neurons into adulthood, the accumulation of *Pitx3*-GFP and *Pitx3*-tauLacZ does not affect the faithful reporting of the Pitx3 protein.

4.1.2 Gain-of-function experiments for ES cells

Gain-of-function analysis by putting transgenes into ES cells has proved to be a valuable system for the study of fate commitment. Gene transfer into mammalian cells may be achieved by infection with a virus that carries the recombinant gene of interest. In this thesis the transgenes were introduced into cells using plasmid DNA. Most of the expression vectors used in this study were derived from pCAGASIP (Appendix I), unless specially notified.

The essential elements of mammalian expression vectors include (1) prokaryotic origin of replication and selection markers for vector propagation in bacteria; (2) a

constitutive or inducible promoter capable of robust transcriptional activity; (3) optimised mRNA processing and transcriptional signals; (4) a eukaryotic selection marker in order to screen the successfully transfected cells (Makrides, 1999). The components of expression vectors used in this thesis are introduced as follows.

The prokaryotic selection box enables the plasmid to be screened and amplified in bacteria *E Coli*. All the plasmids used in the current research contain *Amp*, therefore successful transformants can be selected using 50 μg/ml ampicillin.

Promoters or enhancers: A lot of promoters are available to activate transcription in a ubiquitous or tissue specific manner. The widely used promoters include the human cytomegalovirus (CMV) immediate early promoter, SV40 virus promoter and the murine 3-phosphoglycerate kinase (PGK) promoter. All the expression vectors in this investigation contain the CAG promoter which includes: human CMV immediate early enhancer; chicken β-actin promoter, the untranslated exon 1 and most of intron 1; the 3' portion of intron 2 and part of exon 3 from rabbit β-globin. It is thought that both intron 1 of the β-actin gene and the 3' region of the rabbit β-globin gene contain enhancer-like activity, in addition to the CMV sequence. The CAG promoter can effectively drive high levels of transgene expression in a wide variety of cell lines, including undifferentiated ES cells (Chambers et al., 2003; Chung et al., 2002a; Niwa et al., 2000; Niwa et al., 1991).

Polyadenylation (polyA) signal: The polyA signal is put at the 3' end of target gene cDNA to stop transcription. There are several efficient polyA signals to be used in mammalian expression vectors, including those derived from bovine growth hormone, mouse β-globulin, the SV40 early transcription unit and the Herpes

simplex virus thymidine kinase (HSV *tk*) gene (Makrides, 1999). In this thesis, the bovine growth hormone polyA was put at the 3' end of the *pac* (puromycin N-acetyltransferase) cDNA for puromycin resistance. For inducible expression constructs, polyA derived from HSV *tk* was put after *neo* cDNA.

Internal ribosomal entry site (IRES): IRES is now widely used in many applications that require co-expression of multiple genes from a single transcript by the same regulatory promoter or enhancer (Mountford et al., 1994; Mountford and Smith, 1995). Most expression constructs in this study contain *irespac* (IP) following the gene of interest. Upon transcription, this cassette will create a bicistronic transcript which is translated to make the protein of interest and puromycin N-acetyltransferase. Therefore it is possible to select and maintain cells expressing the gene of interest by puromycin.

All the expression vectors used also contain *ori*, the origin of polyoma virus replication. If the host cells also express the large T protein of polyoma virus, the vectors will be replicated in multiple copies and maintained in episomal status.

ES cell gain-of-function analysis has previously been used for studying mDA neuron differentiation. For example, forced expression of Lmx1a by Nestin-enhancer in ES cells results in generation of TH^+ neurons that all co-express mDA markers, including Nurr1, Pitx3, and En1/2 (Andersson et al., 2006b). Genetic modification of ES cells with the transcription factor Nurr1 leads to increased production of TH^+ DA neurons (Chung et al., 2002b; Kim et al., 2002). Recently, Nurr1 has been shown to induce TH expression in non-neuronal cells in ES cell differentiation (Sonntag et al., 2004), suggesting that Nurr1 controls the biochemical neurotransmitter phenotypes

of DA neurons independent of their neuronal cell fate specification. During my PhD, data in our laboratory have shown that Pitx3 overexpression does not alter the total number of TH^+ DA neurons generated by in vitro differentiation, but enhances the proportion of TH^+ DA neurons that also express the midbrain marker *Pitx3*-GFP (Maxwell et al., 2005). Another group has also reported that Pitx3 overexpression increases the proportion of *Pitx3* expressing cells in the TH^+ population from 21.1% to 92.8% (Chung et al., 2005)

4.2 Derivation of homozygous *Pitx3*$^{GFP/GFP}$ ES cells

4.2.1 Purpose of derivation of homozygous *Pitx3* null ES cells

ES cells with genetic defects may provide useful tools for defining the functional role of particular genes during development, and to study human genetic disease in cellular models (Pera and Trounson, 2004; Wilmut, 2004). We have shown that *Pitx3* is required for maintenance/survival of mDA neurons and the regulation of TH expression (Maxwell et al., 2005). Further work is required to elucidate the detailed molecular mechanisms involved. A homozygous *Pitx3* null ES cell system may provide a platform to evaluate mDA differentiation and maintenance, and to test candidate *Pitx3* downstream targets.

In addition to producing homozygous ES cells where both alleles of the *Pitx3* gene are inactivated, other methods to silence or inactivate the target gene include RNA interference (RNAi) or expression of dominant-negative proteins, which are mutant and inactive forms of the target gene but can interfere with normal protein function (Porter, 1998). The advantage of the latter two methods is that the target gene inactivation is reversible and can be quantitatively controlled. However, the

specificity of RNAi and dominant-negative experiments are not as predictable as knockout studies, and it is necessary to have complementary experiments to confirm that the phenotype is reversed by an expression construct for the target gene in question.

Furthermore, homozygous *Pitx3*$^{GFP/GFP}$ ES cells can also be used to produce chimeric animals to investigate the functional role of *Pitx3* in developing lens and midbrain (Collinson et al., 2004; Rossant and Spence, 1998).

4.2.2 Methods for derivation of homozygous transgenic ES cells

Several methods can be used to produce ES cells that are double knockout. These include sequential targeting of both alleles of the gene of interest (Mortensen et al., 1991; te Riele et al., 1990). In these experiments, inactivation of the targeted gene has been achieved by insertion of a drug resistant gene, so two separate constructs with two different selection markers are required. Another approach is to use a single targeting construct and select the targeted ES cells undergoing spontaneous loss of heterozygosity using high concentrations of drugs (Lefebvre et al., 2001; Mortensen et al., 1992). For sequential gene targeting strategies, there are concerns that the presence of the selectable marker expression cassette, i.e. the drug resistant gene, may influence the expression of neighbouring genes (Nagy, 2000). In our laboratory, the heterozygous *Pitx3*$^{GFP/+}$ ES cells had been established by a targeting construct carrying a floxed PGK promoter-driven hygromycin-thymidine kinase (*hygro-tk*) for selection. The floxed selection cassette has been deleted by transient expression of Cre-recombinase, so the established heterozygous ES cell line is *hygro-tk* negative. Thus it is possible to perform another round of gene inactivation via homologous

recombination using the same targeting construct again. All the methods above use the strategy of gene targeting by homologous recombination, so it is required to screen colonies to confirm their correct integration.

An alternative method could be to derive ES cells de novo from homozygous *Pitx3* null embryos. The expression of *Pitx3* is first detected at E10 (Semina et al., 2000). There is no phenotype of homozygous *Pitx3* null embryos before the blastocyst stage, so it is therefore likely to be possible to generate *Pitx3* null ES cells from homozygous mutant blastocysts. The literature has reported derivation of germ-line competent ES cell lines from denuded preblastocyst mouse embryos when blastomeres are not yet committed to either the inner cell mass (ICM) or trophectodermal lineage (Tesar, 2005). However, it still remains to be further investigated whether these ES cells derived during the preblastocyst period have a wider differentiation potential than those derived from blastocysts. It has also been reported recently that mouse and human ES cells can be derived from single blastomeres by using a technique of single cell embryo biopsy similar to that used in pre-implantation genetic diagnosis (PGD) of genetic defects in IVF clinics (Chung et al., 2006; Klimanskaya et al., 2006). In these experiments each isolated blastomere was aggregated with a small clump of GFP positive mouse or human ES cells, and after incubation on mouse embryonic fibroblast (MEF) feeder cells, the growing GFP negative cells were separated and expanded. Although the authors have claimed that this approach circumvents the ethical concerns about embryo destruction, further evaluation is needed to assess the developmental potential of those parental embryos after biopsy.

The procedure used in this thesis to derive ES cell lines from whole blastocysts on feeders has been described elsewhere (Nichols et al., 1990). It is technically less complicated and the efficiency in generating transgenic ES cells is acceptable.

4.2.3 Derivation of *Pitx3* null ES cells from mutant blastocysts

To obtain *Pitx3* mutant blastocysts, mice were set up to intercross *Pitx3* homozygous with *Pitx3* heterozygous. A total of 9 mice were plugged in 3 days. Mice of the strain 129 were also set up as the control group and 14 of them were plugged. The mice with plugs were injected with Depo-Provera 1 mg subcutaneously and tamoxifen 0.1mg intraperitoneally on E2.5 to delay implantation.

MEF cells, which were used as feeders for ES cell derivation, were exposed to γ-irradiation in order to render them mitotically inactive before being plated into 4-well plates. The medium for the feeder cells was always replaced at least one hour before experiments for pre-equilibration. All mice were sacrificed at E7.5 just before flushing to keep the viability of the blastocysts and a total of 54 blastocysts from the mutant group and 70 from the strain 129 were recovered.

After 5 days the blastocysts attached separately and spread out on the feeder layer whilst the ICMs remained central and began to grow rapidly. The central mass of each explant was picked up for trypsin treatment. Following incubation at 37ºC for 3 minutes, the border of the cell mass began to look rough due to enzyme digestion. Then the cell mass was gently disaggregated into smaller cell aggregates of 3-4 cells, and subsequently transferred to 4-well plates with feeder cells in pre-equilibrated complete medium. Each single ICM-derived clump was disaggregated and plated into one single well of 4-well plates. The dissociated cell clumps began to grow into

different differentiated states, as trophectoderm, primitive endoderm, epithelial-like cells, and primary ES cell colonies. Once they had reached an appropriate colony size, the primary ES cell colonies (Figure 4-1 A) were individually picked and expanded in feeder-free, gelatinised plates (Figure 4-1 B-C). The efficiency of ES line yield from the strain 129 and the mutant group is 16.2% and 13.7%, respectively (p=0.85 by Chi-square test)(Table 4-1). It took one month to establish ES cell lines from blastocysts (Table 4-2).

Since the intercrossing was set up as homozygous x heterozygous, 50% of the blastocysts would be *Pitx3* homozygous (*Pitx3*$^{GFP/GFP}$) and 50% heterozygous (*Pitx3*$^{GFP/+}$). The genomic DNA of the mutant ES cell lines was extracted for PCR genotyping by a combination of three primers (Figure 4-2 A). The *Pitx3*^{GFP} allele produced a band of 590 bp, and the wild type allele a band of 369 bp. Of the 7 lines tested, Pt2A, Pt2B, Pt2C, Pt7B and Pt8C were homozygous *Pitx3*$^{GFP/GFP}$ ES cells; Pt3A and Pt3B were heterozygous *Pitx3*$^{GFP/+}$ ones (Figure 4-2 B).

After disaggregation from primary ES cell colonies, 2 lines (Pt2A and Pt2C) showed a tendency to differentiate. When expanded into feeder-free, gelatinised 6-well plates, only a small percentage of cells remained an undifferentiated state (Figure 4-3 A). To test whether serum-free medium N2B27 plus LIF plus BMP4 (Ying et al., 2003b) could rescue these 2 lines, cells were split equally into 2 parts: one part was cultured in normal ES medium with FCS plus LIF; the other was cultured in N2B27 medium with the combination of LIF plus BMP4 10 ng/ml (R&D). A large proportion of differentiated cells persisted in serum-containing medium (Figure 4-3 B), while in serum-free condition, differentiated cells disappeared gradually and undifferentiated

cells began to dominate (Figure 4-3 D). After 2 passages in N2B27 medium a highly purified population of undifferentiated ES cells were obtained (Figure 4-3 D). When returning to serum-containing medium, they still kept growing in an undifferentiated state, and could be expanded and frozen when appropriate.

The heterozygous line, Pt3A, and the homozygous, Pt2B, were used to produce chimeric animals with wild type morulae via the diploid aggregation procedure. These cells contributed extensively in resulting chimeras as demonstrated by GFP expression in midbrain, developing lens and tongue, thus validating these 2 lines as true ES cells.

	129	mutant
Mice plugged	13	9
Blastocysts flushed out	70	54
Colonies after disaggregration	11	7
Transfer to 6-well dish	11	7
Transfer to small flask	11	6
Established/frozen	10	6
Derivation efficiency	16.2%	13.7%

Table 4-1 ES cell derivation efficiency from blastocysts

9 Feb	Mouse set up	
12	Mouse plugged	Day 3 of set up
14	Depo Provera/Tamoxifene injection	E2.5
19	Blastocysts flush out	E7.5
24	Disaggregation of ICM clumps	5 days after flush
4 Mar	Disaggregation of primary colonies	9 days after disaggregation
7	Expansion into 6-well plate	
9	Expansion into $25cm^2$ flask	
15	Freeze	

Table 4-2 Time required for derivation of ES cells from blastocysts.

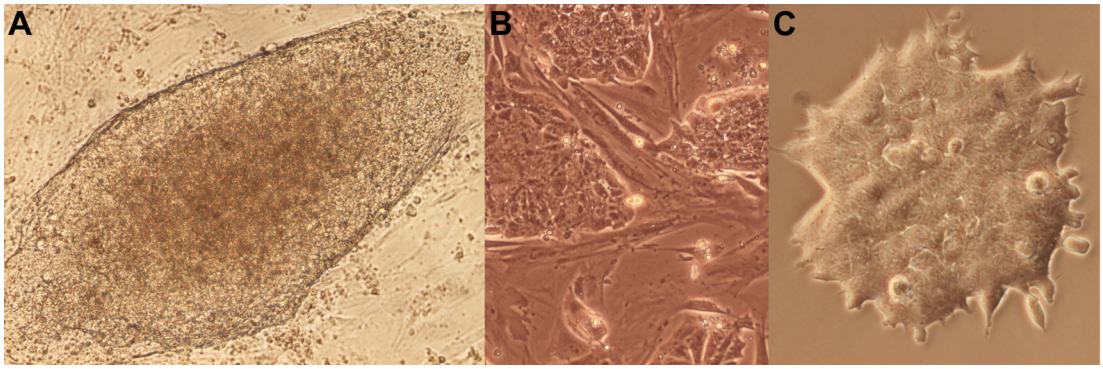

Figure 4-1 Derivation of *Pitx3* null ES cells.

(A) Primary ES cell colony. After disaggregation of primary ES cell colonies, ES cells expanded with (B) and without (C) feeder cells.

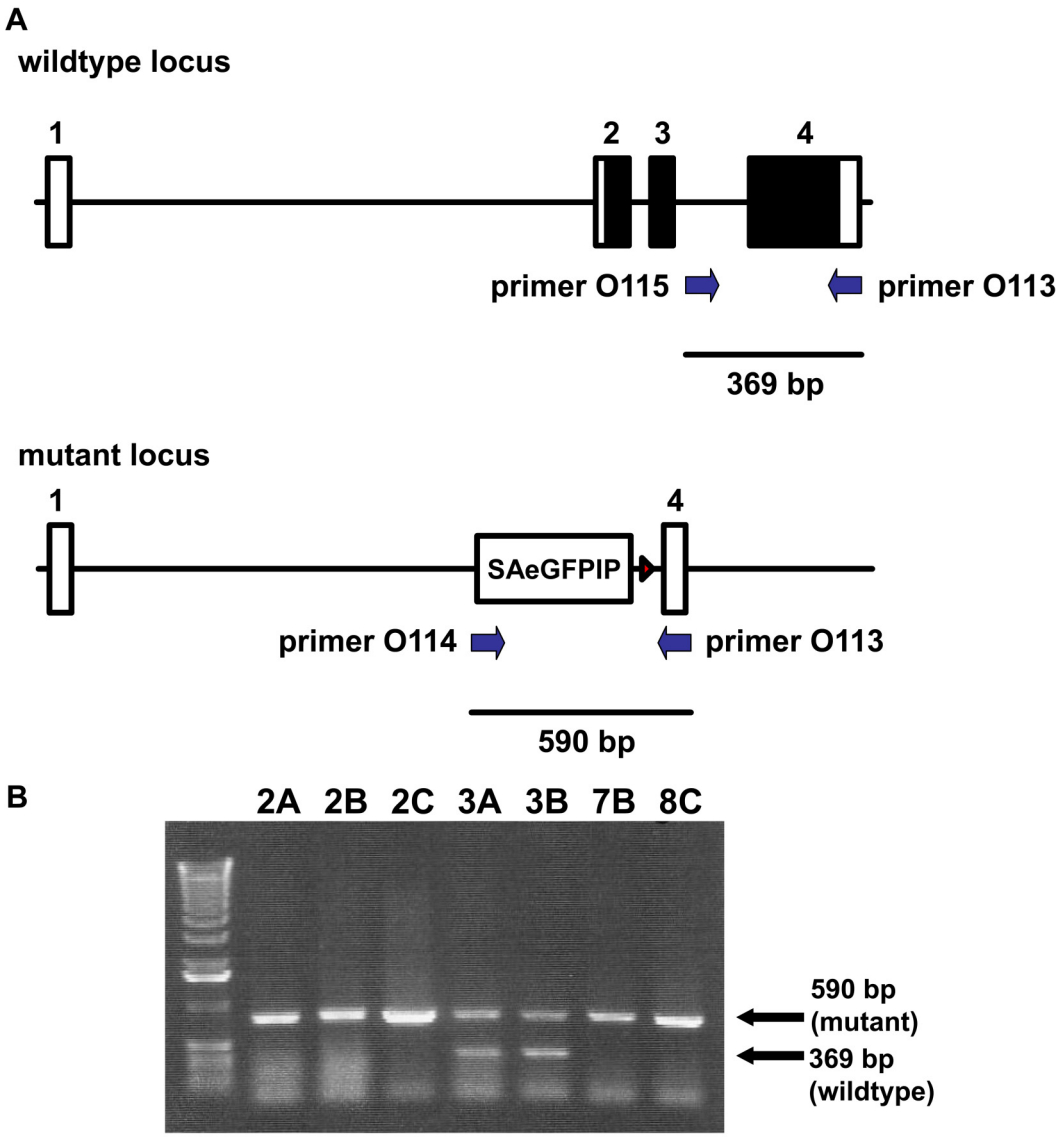

Figure 4-2 PCR analysis for genotyping.

(A) PCR strategy: The homozygous cells should show one band at 590 bp and the heterozygous ones should show 2 bands of 369 bp and 590 bp. (B) In the seven lines tested, 2 were heterozygous and 5 were homozygous.

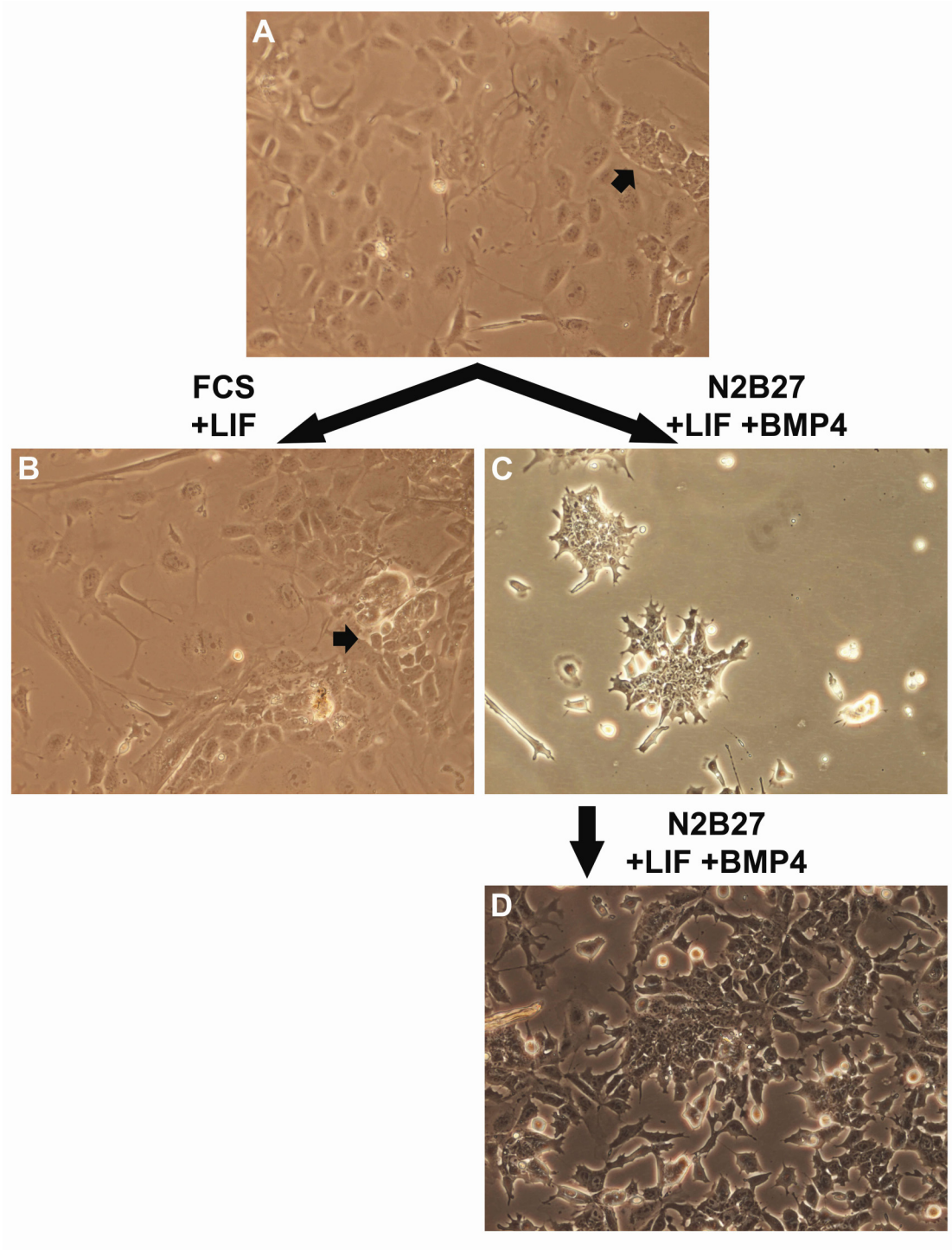

Figure 4-3 Undifferentiated ES cells enriched by serum-free medium.
(A-B) Differentiated cells maintained during culture of Pt2A ES cell lines. (C-D) Undifferentiated ES cells were enriched when cultured in serum-free medium N2B27 with a combination of LIF plus BMP4. Arrow: undifferentiated ES cells.

4.3 Characterisation of *Pitx3*$^{GFP/+}$ ES cell-derived GFP positive cells

ES cells derived were then used for mDA differentiation experiments using the PA6 coculture system. After a total of 2 weeks of in vitro differentiation, *Pitx3*-GFP positive cells with a typical polarised neuronal morphology could be detected. *Pitx3*-GFP was also expressed in other cell types after in vitro differentiation, in keeping with the proposal that in vitro differentiation gives rise to a mixture of cell types, of which the detailed phenotype needs to be further investigated. In this thesis the *Pitx3*-GFP positive cells are referred to as those GFP expressing cells with neuronal morphology.

Most *Pitx3*-GFP positive cells co-expressed β-tubulin III (Figure 4-4 A-C), a marker of neuronal cells, and TH (Figure 4-4 D-F), a marker for DA neurons. Based on expression analysis of *Pitx3*-GFP in developing mouse embryos and adult brain by other members in our laboratory (Zhao et al., 2004), it suggested that these *Pitx3*-GFP positive cells were mDA neurons. However, only 13.5±2.7% (average±SEM) of TH^+ cells co-expressed *Pitx3*-GFP. Since Pitx3 is a specific marker for mesencephalic DA neurons in the central nervous system (Smidt et al., 1997), this data suggested that in vitro differentiation produced a mixture of DA neurons and not all of them display mesencephalic characteristics.

ES cells have the ability to respond to normal developmental cues when introduced into early embryos. However, appropriate inductive signals might not be present during in vitro differentiation. Therefore there are concerns whether the differentiated end cells have the expected patterns of marker gene expression and functional capacity. This led to the requirement to characterise the *Pitx3*-GFP^+ cells using alternative independent mDA antigenic expression. The immunohistochemistry experiments

revealed that the majority of *Pitx3*-GFP$^+$ cells exhibited nuclear distribution of En1 and Nurr1 protein (Figure 4-5), demonstrating their mesencephalic identity.

The *Pitx3*$^{GFP/+}$ ES cells have been used in gain-of-function analysis by other members of this laboratory. It has been shown that Pitx3 overexpression in *Pitx3*$^{GFP/+}$ ES cells produces around 3 times more *Pitx3*-GFP$^+$TH$^+$ cells but does not change the total number of TH$^+$ DA neurons (Maxwell et al., 2005). This led to the question of whether the increased proportion of *Pitx3*-GFP$^+$ cells by Pitx3 was due to terminal fate determination or initiation of ventral mesencephalic neural precursor generation. Therefore, it is necessary to ask whether constitutive expression of Pitx3 in ES cells affect the production of neural progenitors. En1 expression, which is observed in early midbrain neural precursors as well as in differentiated mDA neurons (Alberi et al., 2004), was examined at day 6 of in vitro differentiation when neural progenitors were generated from ES cells. First of all, the differentiation experiment was performed by using 46C (*Sox1*-GFP reporter) ES cells to determine whether En1 expression during in vitro differentiation of ES cells was restricted to neural lineage or not. The transcription factor Sox1 is an early marker expressed in ectodermal cells committed to the neural fate (Pevny et al., 1998), thus the 46C ES cells have proved to be a useful tool in evaluating neural differentiation from ES cells (Aubert et al., 2003; Barraud et al., 2005; Watanabe et al., 2005; Ying et al., 2003b). My immunohistochemistry experiments showed that all En1$^+$ cells co-expressed *Sox1*-GFP (Figure 4-6), suggesting that En1$^+$ cells were of neural cell identity, at least at day 6 in the PA6 coculture system. Nurr1 staining was also evaluated at this stage but was not co-expressed with *Sox1*-GFP at all. Therefore, En1 could be used as a marker to examine midbrain neural progenitors at day 6 of differentiation. Further experiments

showed a similar percentage of En1 expressing cells in the Pitx3 overexpression (9.2±1.1%) and the control (10.3±2.5%) cultures at day 6, suggesting that *Pitx3* overexpression does not appear to have an effect on the generation of mesencephalic DA precursors, and the increased proportion of *Pitx3*-GFP^{+} cells is probably due to terminal differentiation effect (Maxwell et al., 2005).

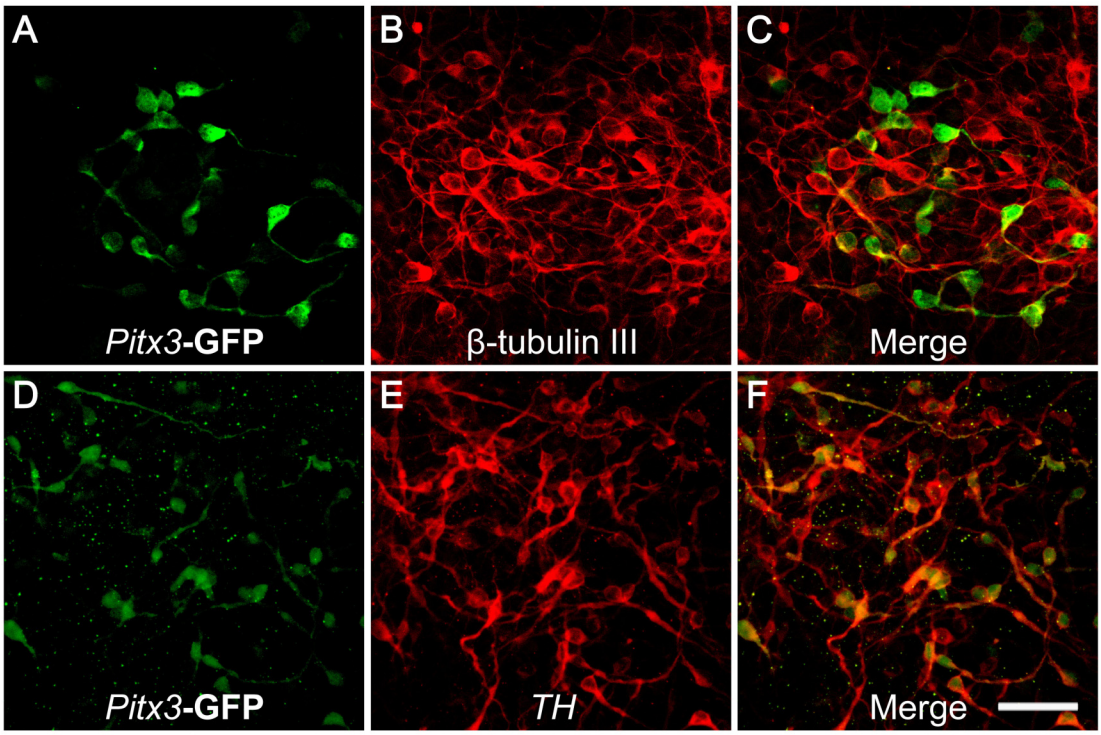

Figure 4-4 β-tubulin III and TH expression of *Pitx3*-GFP^{+} cells

Pitx3$^{GFP/+}$ ES cell-derived GFP^{+} cells with neuronal morphology were detected with GFP distribution in the cell bodies and cytoplasm, including in the axons. Almost all Pitx3-GFP^{+} cells co-expressed cytoplasmic distribution of the neuronal marker β-tubulin III and TH, the rate-limiting enzyme for dopamine synthesis, suggesting that they were dopaminergic neurons.

Scale bar is 40μm.

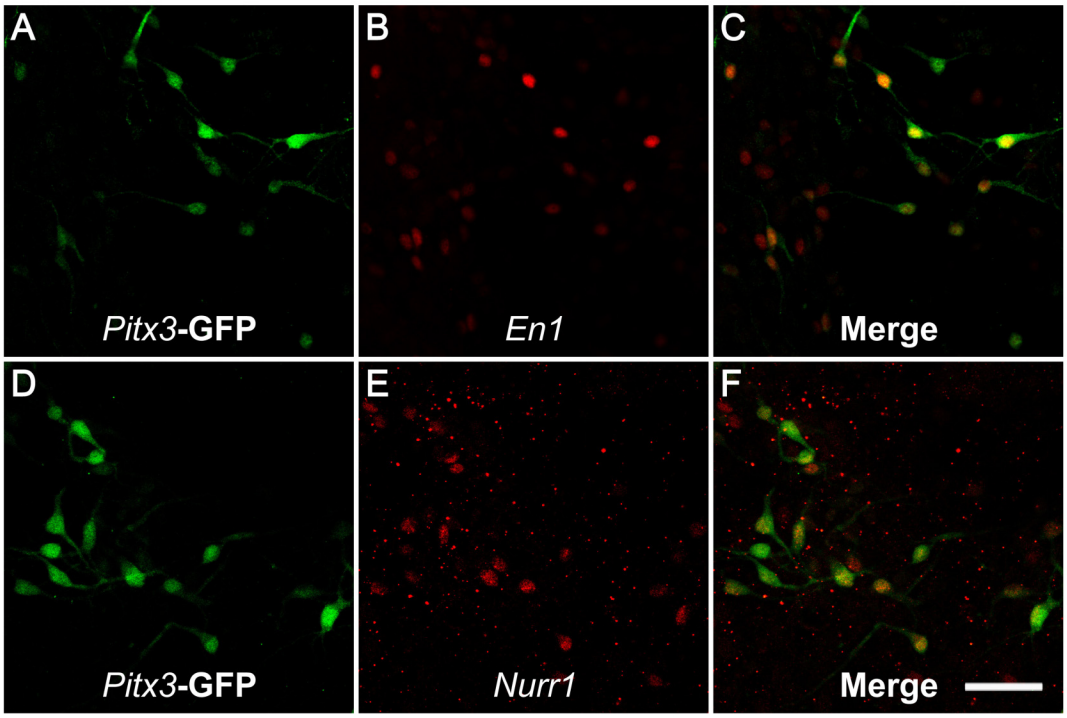

Figure 4-5 *En1* and *Nurr1* expression in *Pitx3*-GFP^{+} neurons.

Nuclear expression of En1 and Nurr1 in the majority of *Pitx3*-GFP^{+} neurons validates their mDA characteristics.

Scale bar is 40µm.

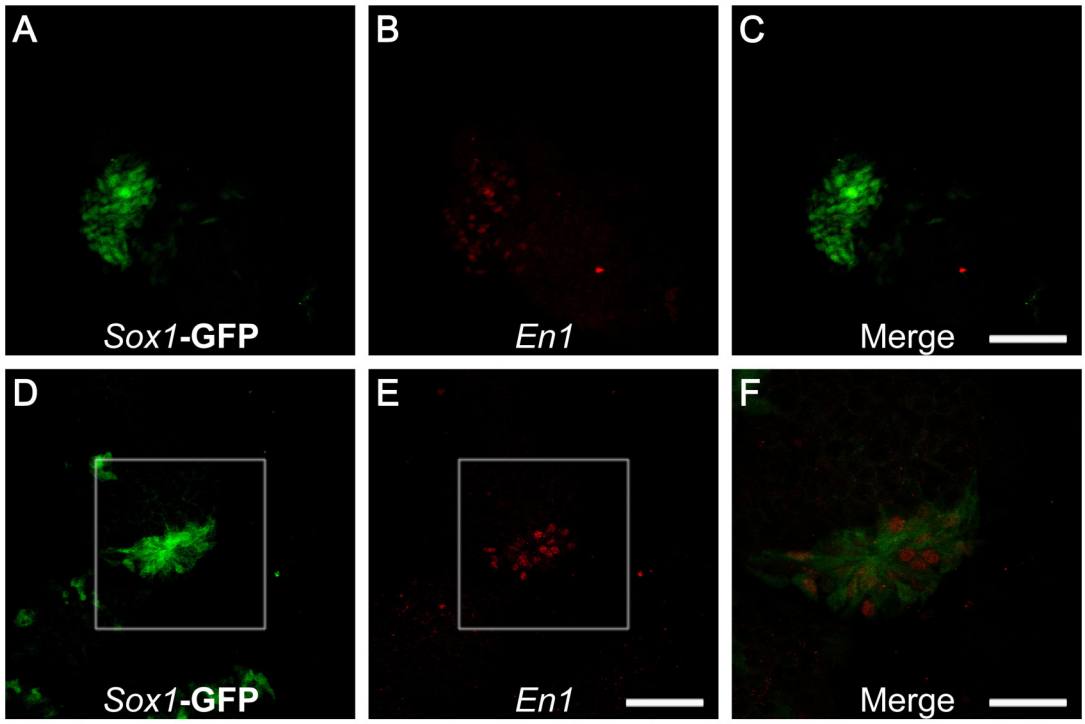

Figure 4-6 En1 expression in *Sox1*$^+$ neural precursors during in vitro differentiation.

At day 6 of in vitro differentiation in PA6 coculture system, nuclear distribution of the En1 protein and cytoplasmic distribution of *Sox1*-GFP was detected in 46C ES cells. All En1$^+$ cells co-expressed *Sox1*-GFP, suggesting that they were neural precursor cells. (F) Inset of D, E.

Scale bars are 80μm for A-C and D-E, 40μm for F.

4.4 In vitro differentiation of *Pitx3*$^{GFP/GFP}$ ES cells into mDA neurons

The GFP reporter can also be exploited to track the fate of *Pitx3* expressing neurons during in vitro differentiation from *Pitx3* null ES cells. With the loss of the Pitx3 protein, GFP$^+$ cells with neuronal morphology could still be detected after 2 weeks of in vitro differentiation. No difference in Morphology could be observed between the *Pitx3* null ES cells and those derived from *Pitx3*$^{GFP/+}$ ES cells (Figure 4-7). The majority of these GFP$^+$ cells co-expressed the neuronal marker β-tubulin III, and mDA markers TH and Nurr1, suggesting that they were mDA neurons. There was no significant difference in the number of TH$^+$ neurons in heterozygous and homozygous *Pitx3*-GFP reporter ES cell lines after in vitro differentiation. Additionally, there was no difference between the percentage of *Pitx3*-GFP$^+$ cells that also expressed TH from heterozygous *Pitx3*$^{GFP/+}$ (92.8±2.2%) and homozygous *Pitx3*$^{GFP/GFP}$ ES cells (92.2±3.4%) (Figure 4-8 A). However, the percentage of GFP$^+$TH$^+$ cells in the TH$^+$ population was 5.8±3.4% in the homozygous group, whilst the percentage of the heterozygous group was 13.5±2.7% (Figure 4-8 B). Thus the proportion of the ES-cell derived TH$^+$ DA neurons that also expressed *Pitx3*-GFP was significantly reduced ($p<0.001$) in the homozygous *Pitx3*$^{GFP/GFP}$ group, compared to the heterozygous *Pitx3*$^{GFP/+}$ group. This suggests a reduction in the proportion of TH$^+$ DA neurons of midbrain dopaminergic type.

A replacement experiment was performed by introducing *Pitx3* expressing vector into *Pitx3*$^{GFP/GFP}$ ES cells to examine whether the impaired potential to differentiate into mDA neurons could be rescued. The *Pitx3* expression construct, pCAGPitx3IP (Figure 4-9 A, Appendix II) has been previously described (Maxwell et al., 2005). Briefly, the *Pitx3* cDNA is driven by the constitutive promoter complex CAG. The

puromycin resistant gene *pac* is linked downstream of the *Pitx3* cDNA via an internal ribosome entry site to ensure that all puromycin resistant cells co-express the *Pitx3* cDNA. A total of 4 lines has been picked up and expanded. The *Pitx3*$^{GFP/GFP}$ ES cells engineered with a Pitx3 transgene will be referred to as PtBR ES cells.

These PtBR ES cells behaved similarly to their parental ES cell lines in standard ES cell culture medium. They did not express *Pitx3*-GFP or TH in the undifferentiated state. The line#1 was used for further differentiation experiments. Following a 2-week in vitro differentiation, PtBR ES cell-derived GFP$^+$ cells exhibited neuronal morphology and also expressed TH (Figure 4-9 B-D). The majority of *Pitx3*-GFP$^+$ cells co-expressed Nurr1 (Figure 4-9 E-G). There was no significant change in the number of TH$^+$ cells generated, but the percentage of TH$^+$ DA neurons that also expressed *Pitx3*-GFP was 47.98±7.29%, which was significantly higher than that were obtained from *Pitx3*$^{GFP/GFP}$ ES cells (5.79±3.43%, $p<0.001$), and even higher than that from heterozygous *Pitx3*$^{GFP/+}$ ES cells (13.5±2.72%, $p<0.001$) (Figure 4-8 B).

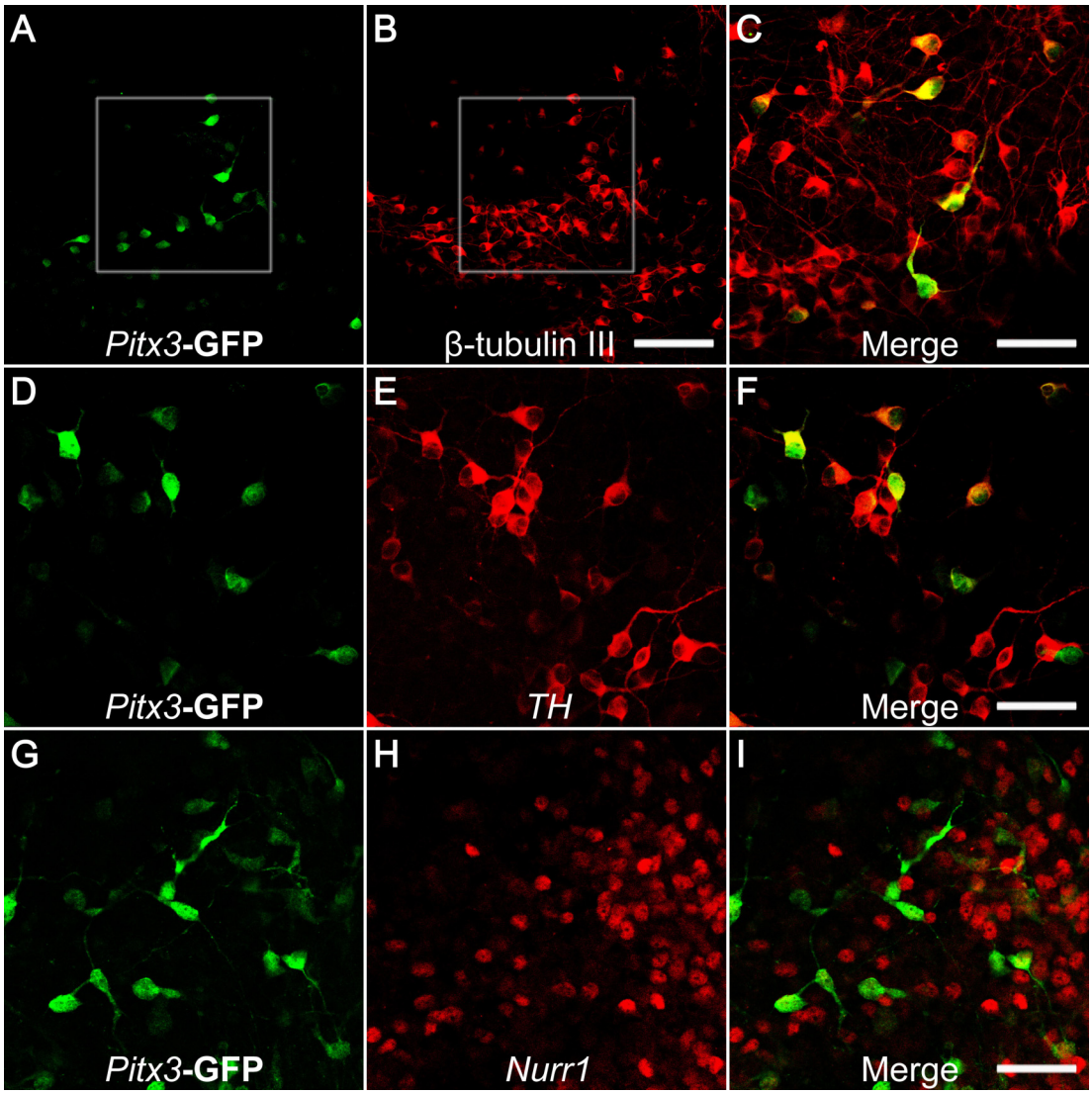

Figure 4-7 Marker expression in *Pitx3*$^{GFP/GFP}$ ES cell-derived GFP^{+} neurons

Pitx3 null ES cells gave rise to GFP^{+} neurons after in vitro differentiation in the PA6 coculture system. Morphologically there was no difference from those derived from *Pitx3*$^{GFP/+}$ ES cells. The majority of these GFP^{+} cells co-expressed the neuronal marker β-tubulin III (A-C) and mDA markers TH (D-F) and Nurr1 (G-I), suggesting that they were mDA neurons.

Scale bars are 80μm for A-B and 40μm for C (inlet of A, B) and D-F.

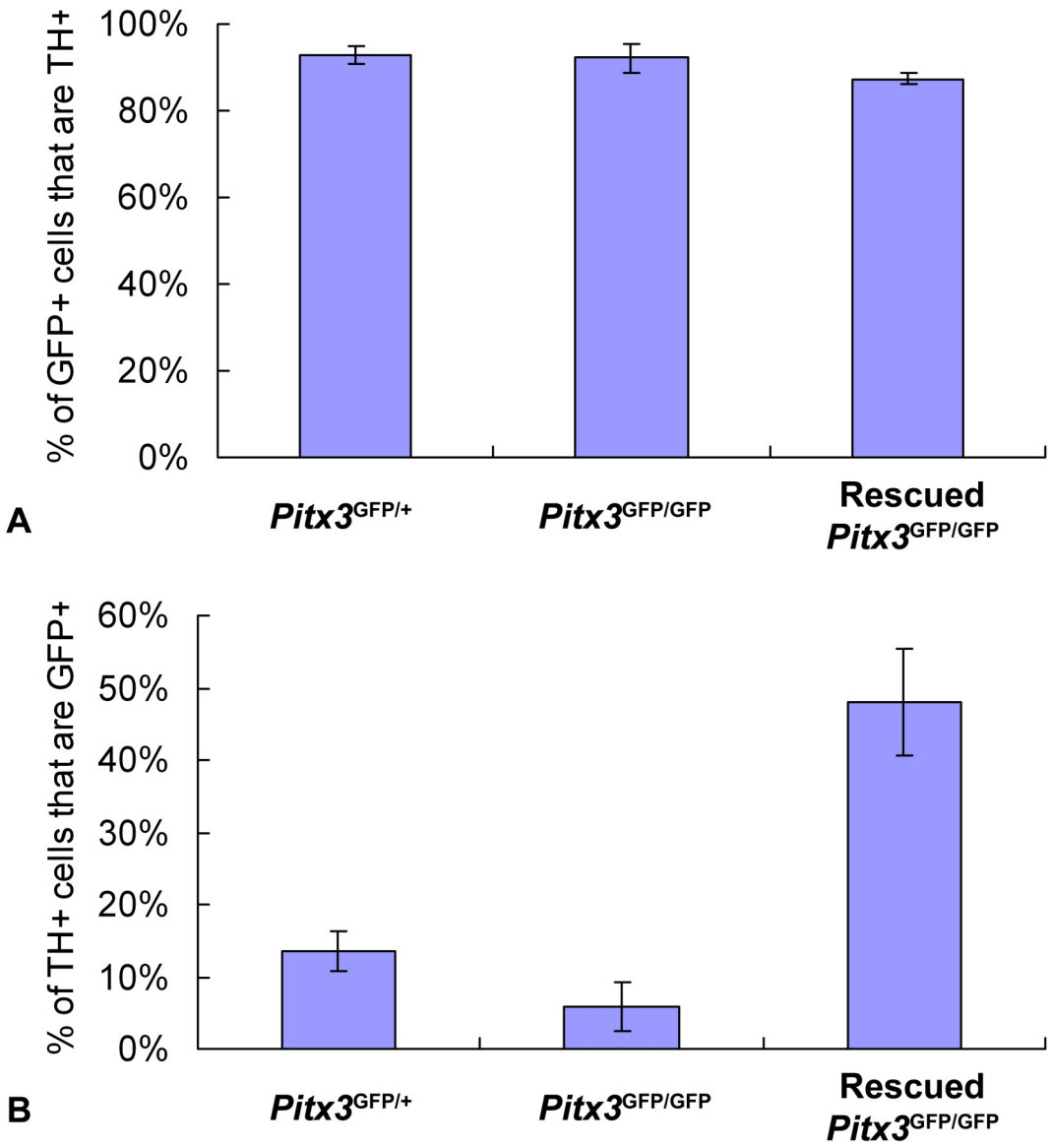

Figure 4-8 Production of *Pitx3*-GFP$^+$ cells from *Pitx3* null ES cells.
(A) The majority of *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ ES cell-derived GFP$^+$ neurons co-expressed TH, suggesting that they are mDA neurons. (B) In the homozygous group, the proportion of TH$^+$ cells that also expressed *Pitx3*-GFP was significantly reduced ($p<0.001$), compared to the heterozygous group. Overexpressing *Pitx3* cDNA in *Pitx3* null ES cells increased *Pitx3*-GFP$^+$ cells in the TH$^+$ DA population.

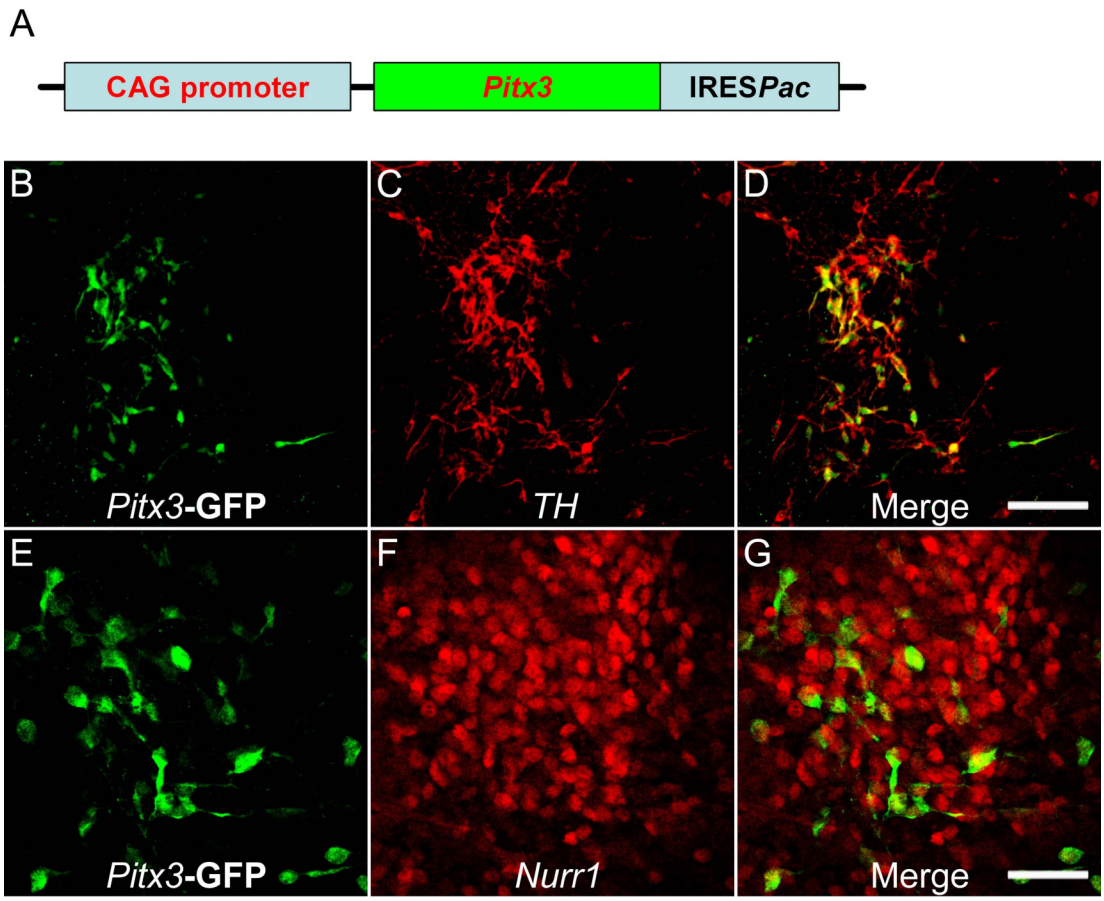

Figure 4-9 In vitro differentiation of rescued *Pitx3*$^{GFP/GFP}$ ES cells.

In the construct of pCAGPitx3IP (A), the *Pitx3* cDNA is driven by the constitutive expression unit CAG and the puromycin resistant gene (*Pac*) is linked downstream of the *Pitx3* cDNA via an internal ribosome entry site (IRES). *Pitx3* null ES cells with replacement of *Pitx3* derived to GFP$^+$ cells after in vitro differentiation. Most of these GFP$^+$ neurons co-expressed mDA markers TH (B-D) and Nurr1 (E-G).

Scale bars are 80μm for B-D and 40μm for E-G.

4.5 En1 overexpression in ES cells during differentiation

The transcription factor En1, a specific midbrain marker for mDA identity, was considered as a candidate regulator of mDA neurons (Alberi et al., 2004; Simon et al., 2001; Wurst et al., 1994). Therefore, I have performed gain-of-function analysis using ES cells to determine whether En1 is involved in mDA specification.

The *Pitx3*$^{GFP/+}$ ES cell line, Pt3A, was used for the En1 gain-of-function test. En1 overexpression construct (Figure 4-10 A, Appendix III) was introduced into *Pitx3*$^{GFP/+}$ ES cells. Since there would be a wide variation in the level of transgene expression, dependent on the chromosomal site of plasmid integration, these puromycin resistant cell lines were checked for protein expression by immunohistochemistry using an antibody against the En1 protein when cultured in 24-well plates. 4 lines (#6, 18, 24, and 29) showed high expression of En1 with nuclear distribution in the undifferentiated state (Figure 4-10 B-D). They were expanded and referred to as PtEn ES cells.

The PtEn ES cells behaved similarly to their parental ES cell lines in standard ES cell culture conditions. They did not express *Pitx3*-GFP or TH in the undifferentiated state. Following 2 weeks of in vitro differentiation, PtEn ES cell-derived *Pitx3*-GFP$^+$ cells with neuronal morphology were detected. These *Pitx3*-GFP$^+$ cells also expressed TH (Figure 4-11), β-tubulin III and Nurr1 (data not shown). Cell counting analysis showed that 13.5±2.72% and 19.72±6.76% of TH$^+$ DA neurons co-expressed *Pitx3*-GFP in the parental and En1 overexpression *Pitx3*$^{GFP/+}$ ES cells, respectively (Figure 4-12). The difference is statistically significant. However, the proportion of GFP$^+$TH$^+$ cells in TH$^+$ DA population by forced expression of the

transcription factor En1 was not enhanced as significantly as other factors (eg. Pitx3), suggesting that En1 does not have a key role in generation or survival of mDA neurons in ES cells.

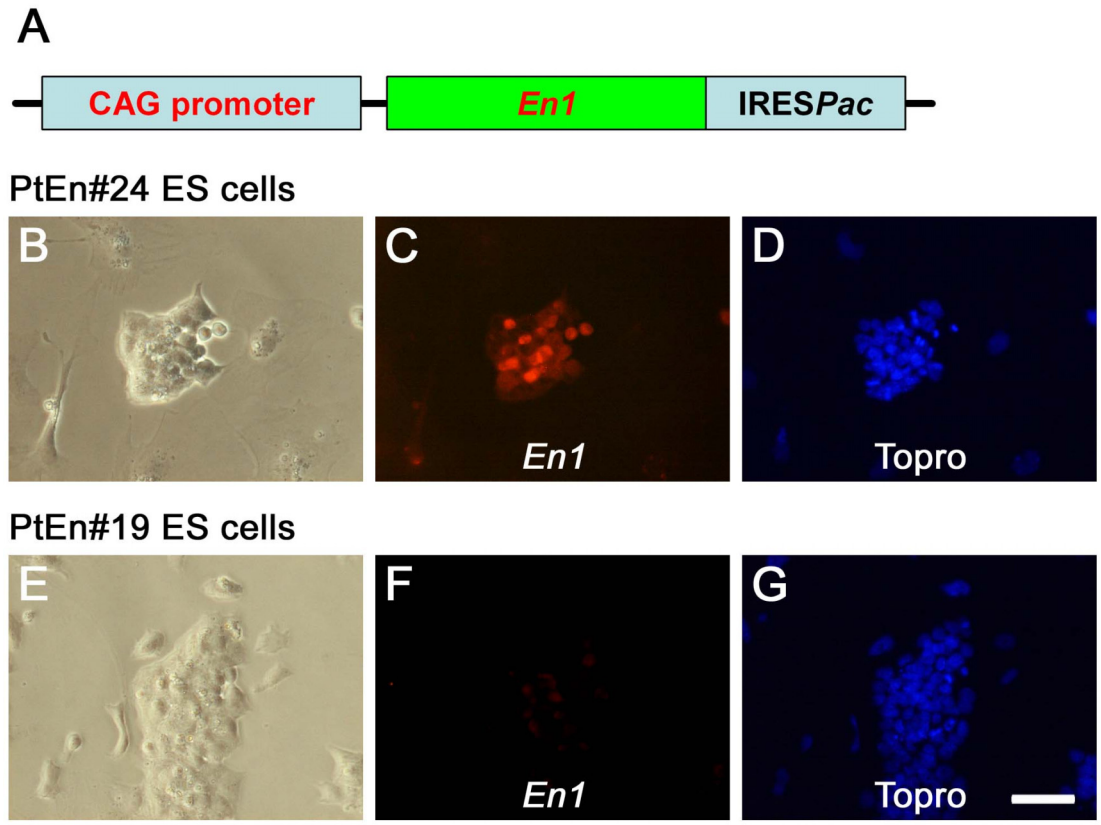

Figure 4-10 *En1* expression in PtEn ES cells.

En1 overexpression construct (A) was introduced into *Pitx3*$^{GFP/+}$ ES cells. Antibody staining showed nuclear distribution of the En1 protein in PtEn ES cells in ES cell state with high (B-D) and low (E-G) expression levels. PtEn ES cells with high En1 expression were used for further differentiation experiments.
Scale bar is 40μm.

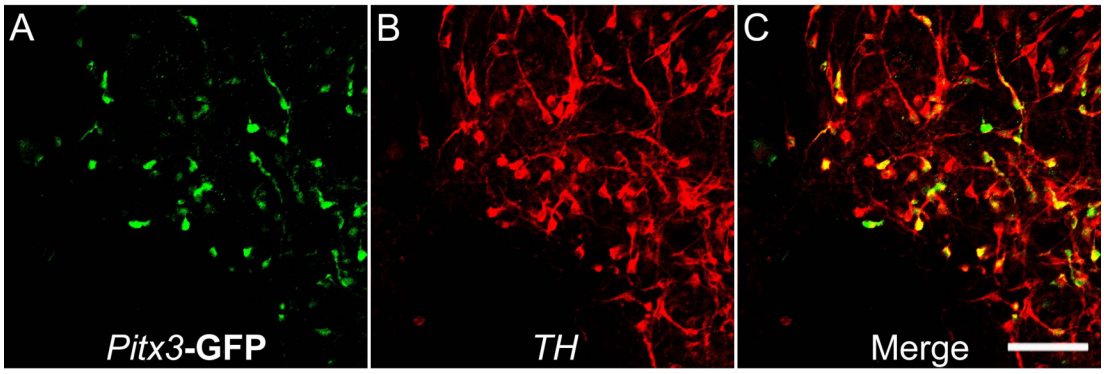

Figure 4-11 In vitro differentiation of PtEn ES cells

Pitx3-GFP^{+} cells differentiated from *En1* overexpression *Pitx3*$^{GFP/+}$ ES cells co-expressed TH, suggesting that they were mDA neurons.

Scale bar is 80μm

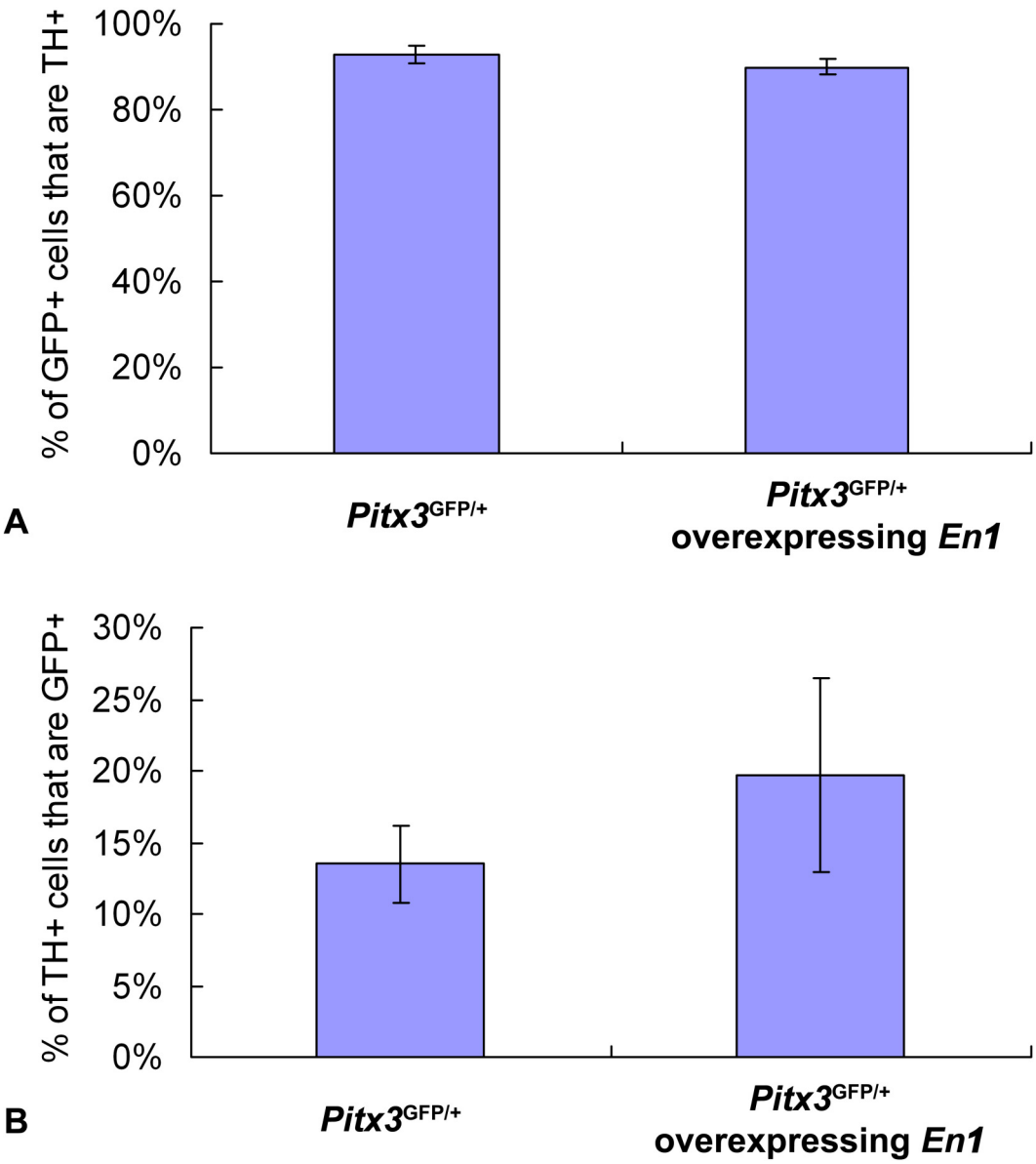

Figure 4-12 Production of *Pitx3*-GFP$^+$ cells from PtEn ES cells.

(A) The majority of PtEn ES cell-derived GFP$^+$ neurons co-expressed TH, suggesting that they were mDA neurons.

(B) When overexpressing En1, the proportion of TH$^+$ cells that also expressed *Pitx3*-GFP$^+$ was increased ($p<0.05$), compared to their parental line.

4.6 Characterisation of a Cre-loxP based ES cell inducible system

4.6.1 Klf4 as a candidate mDA regulator

By microarray analysis, we have identified Krüppel-like transcription factor 4 (Klf4) as a differentially expressed gene in the *Pitx3* positive cell population. Expression levels of *Klf4* in *Pitx3*-GFP positive cells and *Pitx3*-GFP negative cells were 680.0 and 87.2 (measured in fluorescence signal intensities), respectively. Klf4 is also known as gut-enriched Klf (GKLF), and was initially identified as an epithelially enriched gene with preferential expression in the terminally differentiated, post-mitotic epithelial cells of the intestine and epidermis (Shields et al., 1996). In the intestinal epithelium, *Klf4* is expressed in the upper region of the crypt, in which cells have undergone growth arrest and begun to differentiate into mature colonocytes (Shie et al., 2000; Shields et al., 1996). Mice homozygous for a null mutation in *Klf4* die shortly after birth and show selective perturbation of the late stage differentiation structures of the epidermis, resulting in loss of skin barrier function (Segre et al., 1999). *Klf4* homozygous mutant mice also show disturbed terminal differentiation of goblet cells in the colon (Katz et al., 2002). In cultured cells, expression of *Klf4* is associated with conditions that lead to growth arrest such as serum deprivation or contact inhibition. In addition, Klf4 is also known as a tumour suppressor gene and its expression is decreased in conditions that involve increased proliferation such as neoplasm of the intestinal tract and in cancer cell lines (Rowland et al., 2005; Rowland and Peeper, 2006; Shields et al., 1996).

On the other hand, the functional role of Klf4 in other tissues remains elusive. It is worth noting that *Klf4* overexpression in ES cells promotes self-renewal and inhibits

ES cell differentiation (Li et al., 2005). This is because *Klf4* expression sustains *Oct4* expression in ES cells (Nakatake et al., 2006). A recent report demonstrates that introducing *Klf4*, in combination with *Oct3/4*, *Sox2* and *c-Myc*, induces mouse embryonic and adult fibroblasts into pluripotent stem cell-like cells exhibiting the morphology and growth properties of ES cells and expressing ES cell marker genes (Takahashi and Yamanaka, 2006), supporting the proposal that Klf4 is important molecule for self-renewal in ES cells.

Further validation in our laboratory by RT-PCR and RNA in situ hybridisation confirmed differential expression of *Klf4* in *Pitx3*-GFP positive cells compared to *Pitx3*-GFP negative cells in the E14 midbrain (Figure 4-13). Since most functional studies of Klf4 show that it is required for terminal differentiation by regulating the cell cycle, it is interesting to investigate whether *Klf4* is also involved in mDA differentiation. However, constitutive expression of *Klf4* in ES cells precludes differentiation and promotes self-renewal (Li et al., 2005; Takahashi and Yamanaka, 2006). Therefore, a temporally controlled activation of *Klf4* is necessary in the ES cell gain-of-function experiment for mDA development. This led to the development of an inducible *Pitx3*$^{GFP/+}$ ES cell system.

4.6.2 Inducible gene expression in ES cells

If a gene of interest has multiple roles and acts during different stage of ES cell differentiation, constitutive expression of such a molecule in ES cells may lead to confusing outcome and preclude a precise evaluation of its functional role in later stages of development. Conditional expression allows such multiple or stage dependent roles to be investigated in ES cells.

An ideal inducible system should fulfil two requirements, specificity and efficiency. For specificity, the system should not respond to endogenous factors and should be activated only by exogenous drugs that do not interfere with cellular regulatory pathways. For efficiency, the basal activity of the system (leakage) should be minimal, while in the active state it should quickly generate high levels of gene expression. Currently conditional transgenesis can be achieved by transcriptional transactivation, which may be induced by tetracycline, rapamycin, mifepristone (RU486) and ecdysone (Rossi and Blau, 1998; Saez et al., 1997). An alternative system is based on the site-specific DNA recombination by Cre recombinase.

The bacteriophage P1 Cre (cyclisation recombination) recombinase recombines DNA at the 34-bp sequence called loxP (locus of crossover of P1). If the loxP sites are placed in the same orientation on a strand of DNA, the Cre recombinase efficiently excises DNA flanked by the two loxP sites (floxed) in mammalian cells (Nagy, 2000). Feil et al. have fused Cre recombinase to a mutant (G525R) ligand-binding domain (LBD) of human oestrogen receptor (ER) in order to generate the chimeric recombinase CreER^{T}, whose activity in cultured cells is dependent on the presence of the synthetic ligand 4-hydroxy-tamoxifen (4OHT), rather than endogenous oestrogen (Feil et al., 1996). Short-term 4OHT treatments have very little toxicity to cultured cells. The later developed CreER^{T2}, which contains triple mutation (G400V, L539A and L540A) to the human ER (Feil et al., 1997), is 10-fold more sensitive than CreER^{T} in a dose-responsive recombinase activity (Indra et al., 1999). It has also been claimed that there is undetectable background activity of Cre recombinase in the absence of 4OHT (Feil et al., 1996; Indra et al., 1999).

To achieve conditional transgene expression, a floxed 'transcription stop sequence' has to be placed between a gene promoter and the transgene so that transcription of the transgene does not proceed until Cre recombinase excises the stop sequence. The 'transcription stop sequence' can be served by a polyadenylation (polyA) signal constructed following cDNA of a reporter or selection gene. This strategy allows the establishment of transgenic cell lines with a silent genetic alteration that can be activated by Cre-mediated excision.

4.6.3 Principle of PtCreER ES cell system

Our laboratory have developed an inducible system for conditional gene expression in *Pitx3*$^{GFP/+}$ ES cells that relies on 4OHT-dependent Cre recombinase-mediated activation. There are two important components in the inducible system: the PtCreERT ES cells which are *Pitx3*$^{GFP/+}$ ES cells constitutively expressing CreER^{T2}, and the inducible construct which contains a floxed stop signal (*neoPA*) between CAG promoter and the transgene cDNA (Figure 4-14).

The CreER^{T2} construct, which was kindly provided by Lars Grotewold in Austin Smith's laboratory, was put into the *ROSA26* locus of *Pitx3*$^{GFP/+}$ ES cells. The *ROSA26*, located at chromosome 6, is a gene locus from which proteins can be expressed ubiquitously at a moderate level (Zambrowicz et al., 1997). Therefore, these cells constitutively express the fusion protein CreER^{T2}. They are referred to as PtCreERT ES cells. The CreER^{T2} is retained and sequestered in the cytoplasm until 4OHT administration which allows nuclear localisation and the site-specific recombination.

Various doses of 4OHT were used to test its toxicity in ES cell culture conditions. After 24 hours of culture, ES cell numbers were counted and compared with the minus 4OHT control group. As shown in Figure 4-15, concentration of 4OHT up to 1 uM did not show detrimental effects on ES proliferation/maintenance.

The construct pCAGfloxedNeoIP (Appendix IV) is available to put the candidate genes in the region between the floxed *neoPA* and *irespac*. The neomycin resistant gene (*neo*) and the following polyA signal, which is derived from HSV thymidine kinase, is floxed by two loxP sites. After introduction into the PtCreERT ES cells, the cells in which the plasmid is successfully integrated can be selected for with G418 because *neo* is driven by the CAG promoter. The transgene is silent at this time because there is a polyA signal in front of it (Figure 4-14 A). Following activation of the Cre recombinase by 4OHT, the floxed *neoPA* will be deleted and the cells will become G418 sensitive, puromycin resistant and the transgene cDNA will be expressed (Figure 4-14 B).

4.6.4 Characterisation of induction in ES cell status

A control experiment was performed to test the efficiency of induction for transgene expression using an inducible vector which contains the GFP reporter following the floxed *neoPA*. The PtCreERT ES cells with stable integration of this plasmid are GFP negative. However, GFP is expected to be activated following Cre-mediated excision of the floxed *neoPA*.

4 lines of PtCreERT ES cells were tested for proper inducibility of GFP expression. In the absence of 4OHT induction, GFP expression was detected in a very small percentage of cells in all 4 lines tested (Figure 4-16 A). Flow cytometry analysis

revealed that this leakage rate of transgene expression was 0.95%, 0.64%, 0.89% and 1.39%, for each of the four lines.

24 hours after the addition of 1 μM 4OHT, the majority of cells in 3 of the 4 lines tested expressed GFP (Figure 4-16 B). Flow cytometry analysis for the proportion of GFP^+ cells in the total population showed that the induction efficiency after 24 hours of treatment was 17.60%, 92.38%, 96.37% and 93.40%, respectively (Figure 4-16 C). Therefore, these cells showed low basal levels of expression in the uninduced state and rapidly achieved high levels of expression upon induction.

For a more detailed time and dosage relationship in ES cell status, the PtCreERT#3 ES cells carrying the GFP inducible vector were treated with various doses of 4OHT with various durations of treatment (Figure 4-17). I found that 12 hours of treatment with 0.05 μM 4OHT was sufficient to induce GFP expression in the majority of cells. Higher concentrations or longer treatment did not further improve induction efficiency, at least in undifferentiated ES cell cultures.

4.6.5 Characterisation of transgene induction during neural differentiation

Induction efficiency was also examined during in vitro differentiation by the monolayer differentiation method. PtCreERT#3 ES cells, carrying the inducible vector containing the GFP reporter, were plated on gelatinised 6-well plates with a density of 5 x 10^3 cell/cm^2. At day 3 of monolayer differentiation, various doses of 4OHT were added to induce the transgene expression. After 24 hours of treatment, flow cytometry analysis revealed that 4OHT dosage higher than 0.5 μM did not induce more cells to express GFP (Figure 4-18). However, the induction efficiency of 24 hours of treatment at day 3 of monolayer differentiation was ~87.61%, less than

that of ES cell status (96.54%).

The lower induction rate during differentiation could be caused by lower efficiency of the Cre-based induction process or lower CAG promoter activity. To clarify this question, I divided the induction experiments into 4 groups at day 3 of monolayer differentiation: (a) 48 hours of 0.5 μM 4OHT treatment, (b) 24 hours of 4OHT treatment followed by another 24 hours without 4OHT, (c) 24 hours without 4OHT followed by 24 hours of 4OHT treatment, and (d) 48 hours of minus 4OHT as a control (Figure 4-19 A). Flow cytometry analysis on day 5 of differentiation showed that 48 hours of 4OHT treatment resulted in a high induction efficiency of ~93.61% which was comparable to that observed in undifferentiated ES cell status. Interestingly, the same level of induction efficiency could be obtained if 4OHT was added in the first 24 hours then removed in the following 24 hours, suggesting that the first 24 hours of induction was sufficient to delete the floxed sequence in the majority of cells. These data demonstrated that the $CreER^{T2}$ system is as efficient during differentiation as in undifferentiated ES cells, but more culture time is needed for the CAG promoter to drive transgene expression during differentiation. However, a further 24 hour culture following a 24 hour 4OHT induction allows maximum GFP expression during monolayer differentiation (Figure 4-19 B).

In summary, the combination of *ROSA26* driven $CreER^{T2}$ and the floxed-stop transgene vectors driven by CAG promoter is an efficient inducible system for transgene expression. In ES cell status, 0.05 μM 4OHT for 12 hours of treatment is sufficient to obtain the maximal expression of induced transgene. During monolayer differentiation, 0.5 μM 4OHT for 24 hours is sufficient to delete the floxed stop

signal and to activate the transgene.

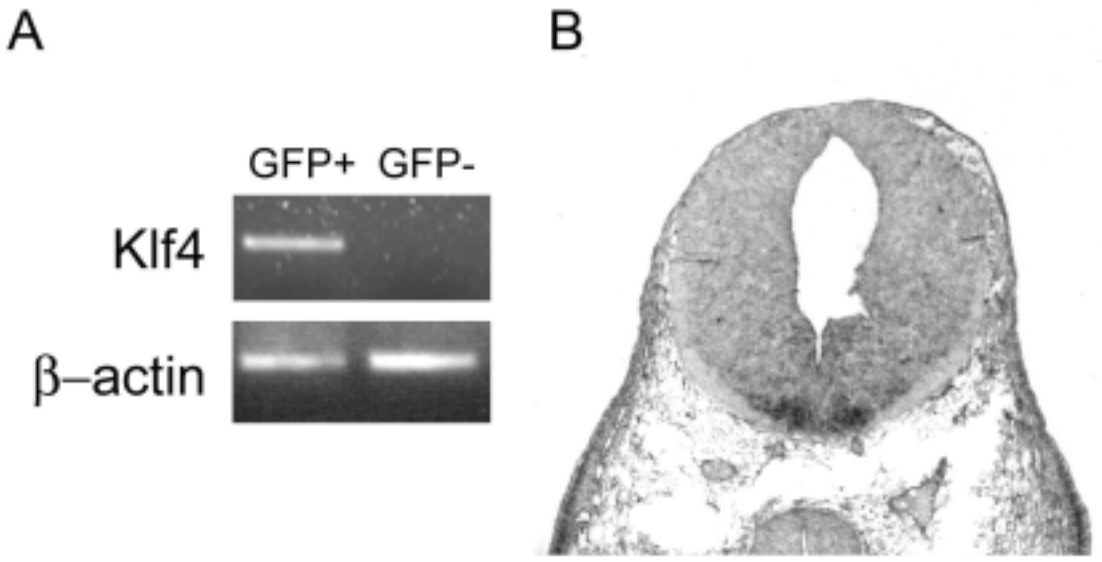

Figure 4-13 Klf4 validated as a candidate mDA regulator

(A) *Pitx3*-GFP^{+} and *Pitx3*-GFP^{-} cells in the E14 midbrain were isolated by FACS and RT-PCR showed differential expression of *Klf4* in *Pitx3*-GFP positive cells (data kindly provided by Joaquim Vives). (B) RNA in situ hybridisation revealed the expression of *Klf4* at E10 in the ventral midbrain (data kindly provided by Suling Zhao).

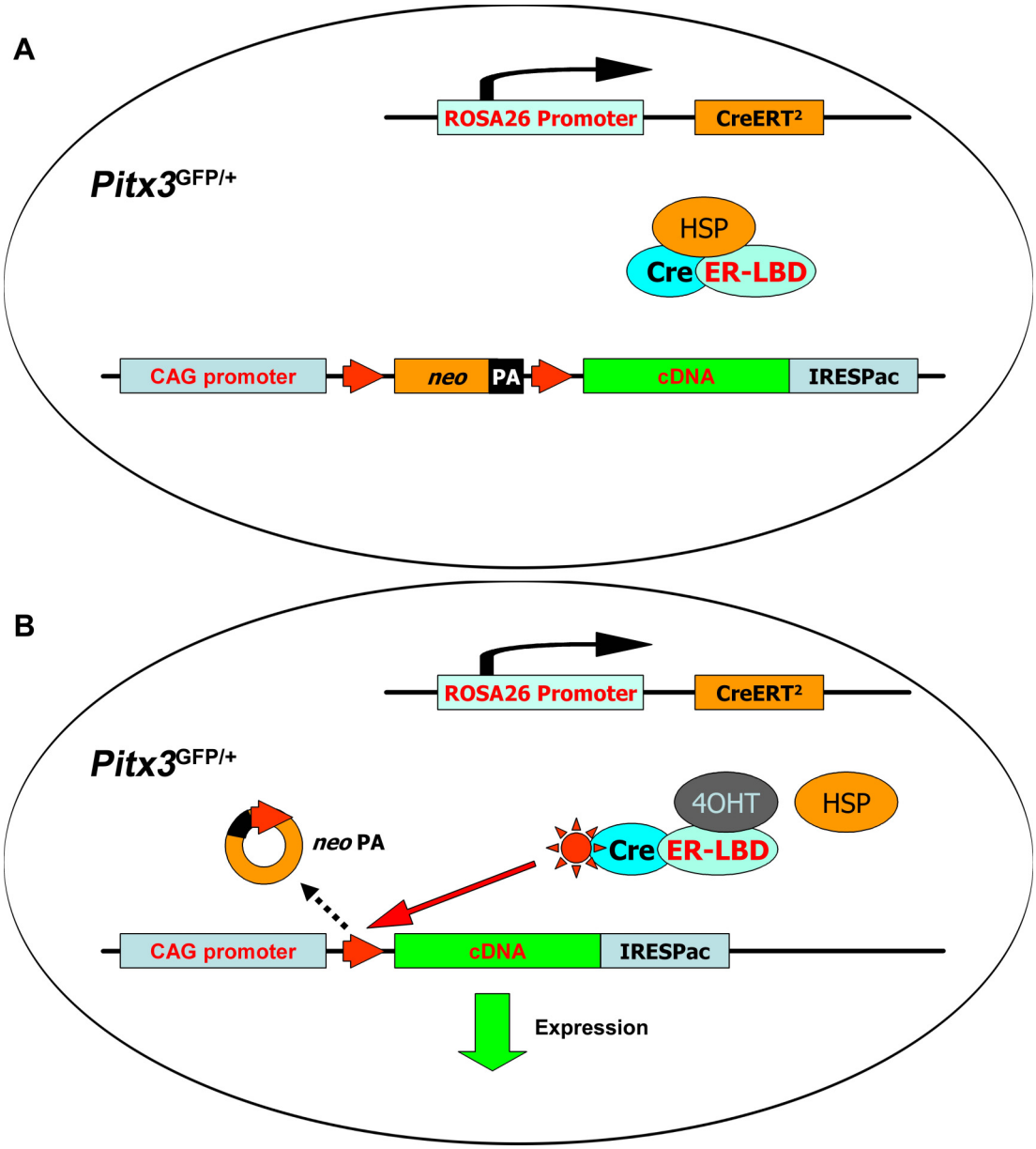

Figure 4-14 Principle of *Pitx3*$^{GFP/+}$ inducible system.

(A) In the PtCreERT ES cells, the fusion protein CreER^{T2} is constitutively expressed by the ROSA26 regulatory elements. The transgene cDNA can be inserted downstream of the floxed *neoPA*, which prevents the transcription of the transgene cDNA. (B) With the addition of 4OHT, the Cre recombinase will be activated and will excise the floxed stop signal simultaneously bringing the transgene cDNA under CAG promoter control.

HSP: heat shock protein; ER-LBD: oestrogen receptor-ligand binding domain.

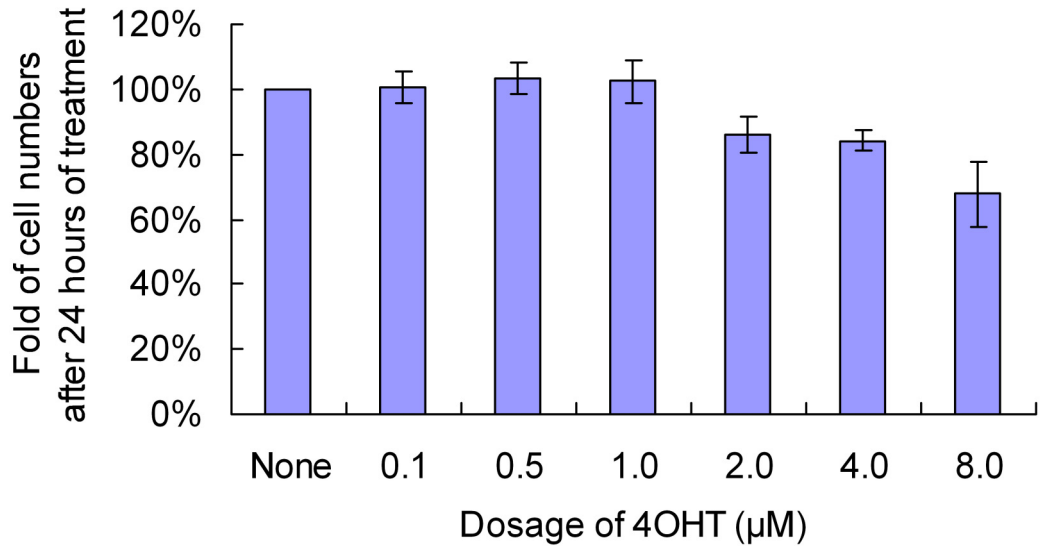

Figure 4-15 Toxicity of 4OHT for ES cell proliferation

PtCreER ES cells were plated at the same cell density with the addition of various doses of 4OHT and compared with the control group after 24 hours of culture in ES cell status. 4OHT concentration up to 1 μM showed no detrimental effects on ES cell proliferation.

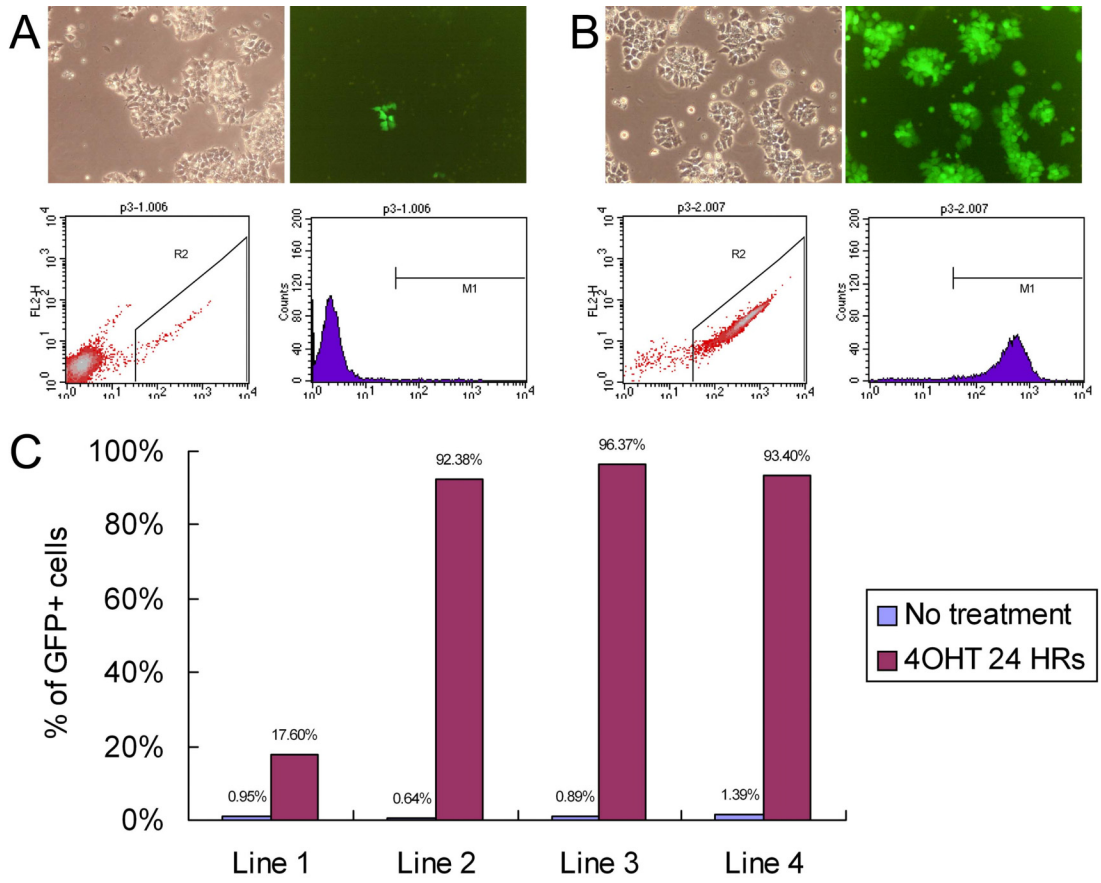

Figure 4-16 Induction efficiency of 4 PtCreERT lines

The inducible plasmid carrying GFP reporter was inserted into 4 PtCreERT ES cell lines to test the induction efficiency. The percentage of GFP^+ cells was determined by FACS. Without induction, a very small percentage of cells expressed GFP (A). Twenty-four hours after treatment with 1 μM 4OHT, the majority of cells in the line PtCreERT#3 expressed the GFP reporter (B). PtCreERT#2, #3 and #4 showed a low leakage of transgene expression before treatment and high induction efficiency after treatment with 4OHT (C).

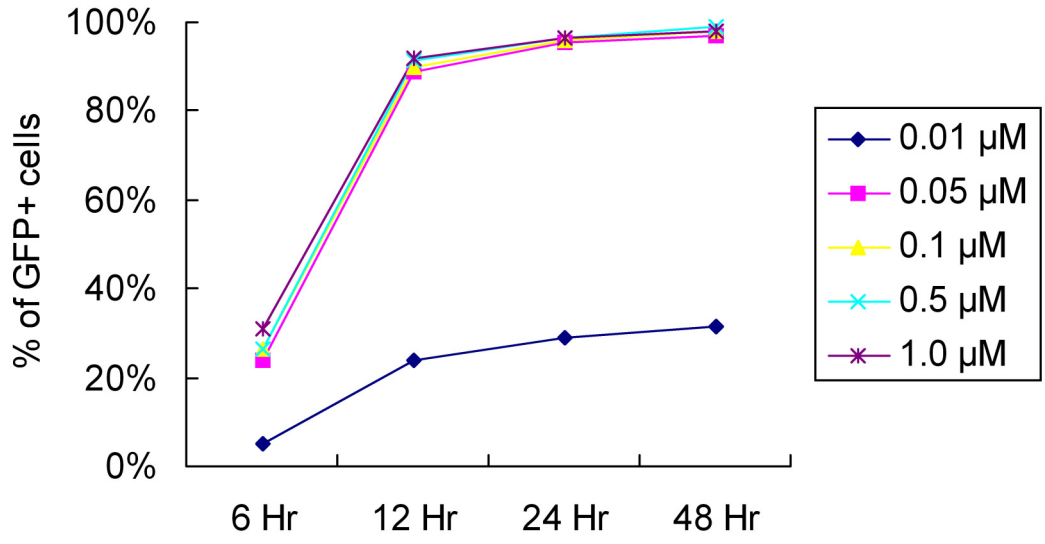

Figure 4-17 Time-dosage characterization of induction in ES cell state. PtCreERT#3 ES cells were treated with various doses of 4OHT for differing durations. 0.05 μM 4OHT treatment for 12 hours is sufficient to induce transgene expression in ES cell status.

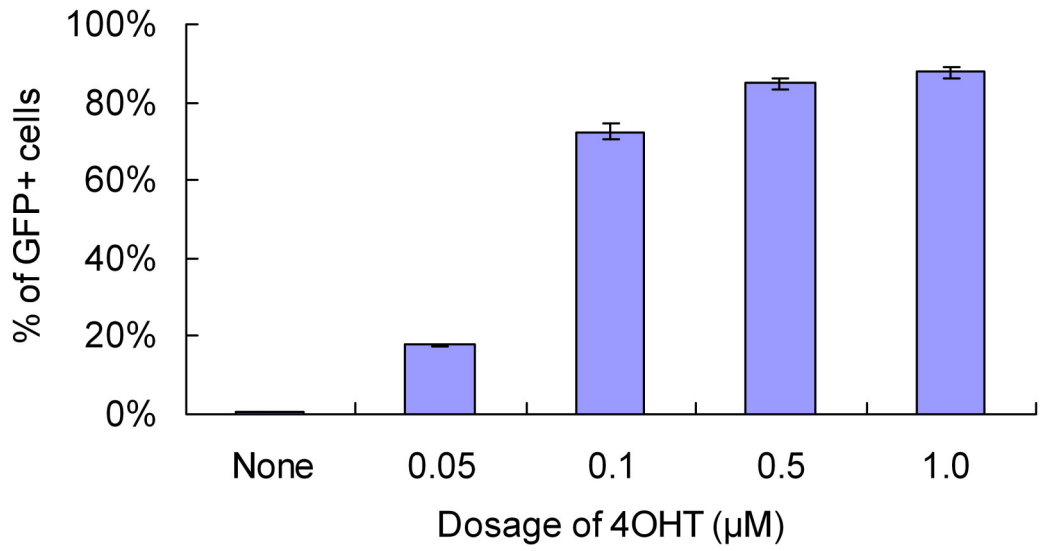

Figure 4-18 Dosage titrations for induction during monolayer differentiation.

Various doses of 4OHT were used to induce transgene expression at day 2 of monolayer differentiation. After 24 hours the percentage of GFP^+ cells was obtained for flow cytometry analysis.

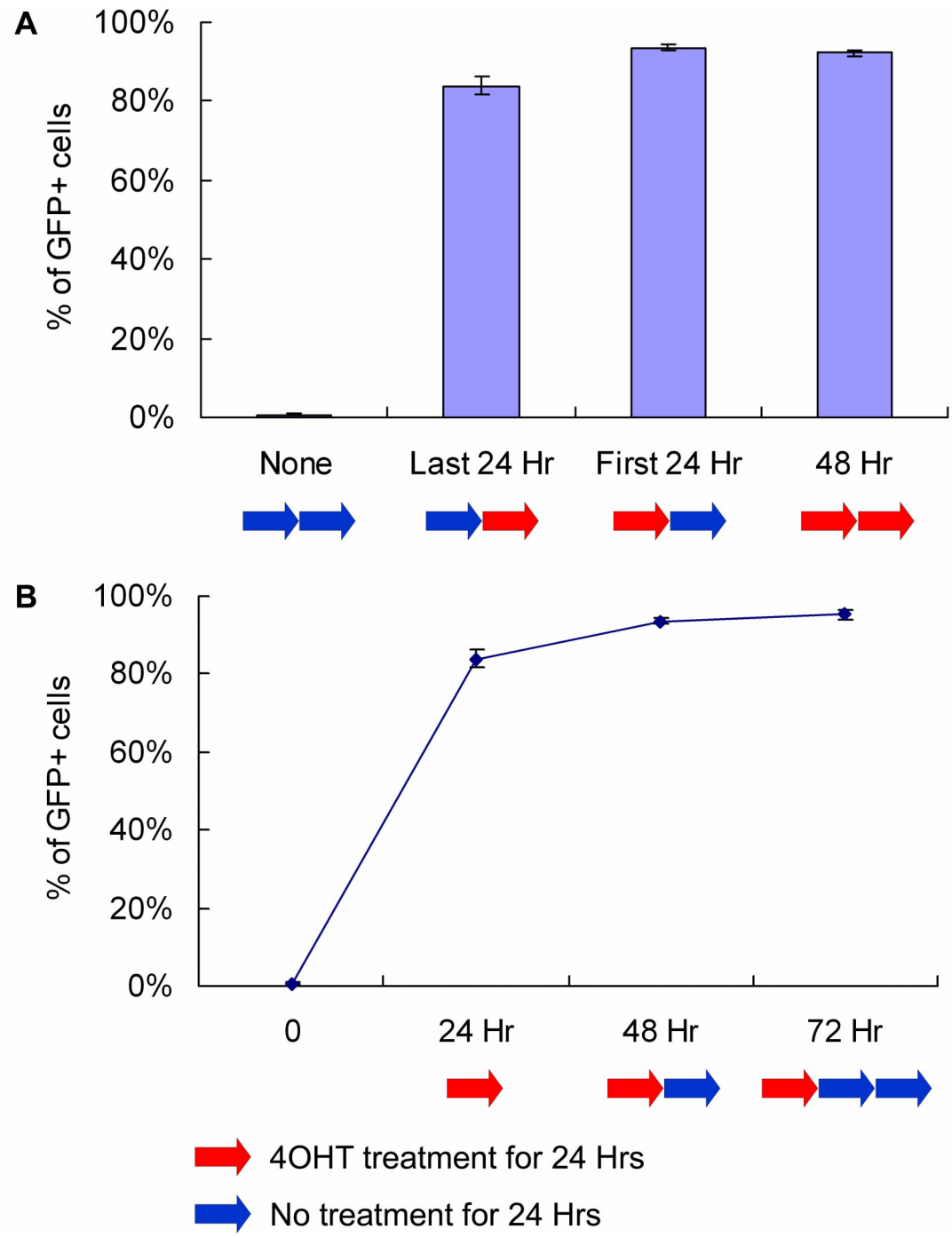

Figure 4-19 Time characterisation of induction efficiency during monolayer differentiation.

(A) 24 hours of 4OHT treatment is sufficient for maximal induction efficiency. (B) 48 hours is sufficient for the induced transgene to be expressed during monolayer differentiation.

4.7 Episomal gene expression system

By microarray analysis, other members in our laboratory are searching for mDA regulators. It is helpful to establish an ES cell system to screen the candidate genes in an efficient manner. The polyoma-based episomal replication system allows maintenance of exogenous DNA sequences in episomal plasmids, that is, as non-integrated circular DNA. The cells in this system are easily transfected and provide a means for rapid validation of candidate genes (Aubert et al., 2002; Chambers et al., 2003).

4.7.1 Introduction for episomal gene expression system

- Polyoma virus and the viral-derived vector

The original polyoma viral genome contains a circular, double-stranded DNA of 5.3 kb. The genome is functionally divided into an early region, which is transcribed and expressed early after the virus enters the cells; a late region, which encodes for the viral capsid proteins; and a noncoding regulating region. The early region encodes for: (a) large tumour (T) antigen, a 100 kDa nuclear protein essential for initiating viral DNA replication; (b) middle T, a 48 kDa protein involved in cellular transformation; and (c) small T, a 22 kDa protein of unknown function. These proteins are generated from alternative splicing of the same transcript. Stable replication of polyoma-derived vectors within transfected cells requires a functional polyoma origin of replication (*ori*), large T protein with its intact DNA-binding, helicase activity and the set of cellular proteins involved in replicating DNA synthesis.

The viral part of the vector pMGD20neo (Appendix VII) consists of the polyoma *ori*;

a modified early regulatory region, which lacks the splice sites for processing the middle and small T mRNAs; and a mutant enhancer region, PyF101, which was originally selected for the ability to replicate the polyoma genome in embryonic carcinoma cells (Fujimura et al., 1981). This plasmid also contains a eukaryotic selection cassette, the neomycin resistance gene driven by PGK promoter (*PGKneo*), thus it is possible to screen the transfected cells by G418 selection. The vector pMGD20neo is unable to transform host cells because it cannot express middle T protein, which is responsible for viral oncogenic transforming activity.

It has been shown that pMGD20neo can be maintained episomally at about 10-30 copies per cell for at least 74 ES cell generations in the presence of G418 (Gassmann et al., 1995). Removal of selective pressure does not affect the episomal status of the polyoma-derived vectors, suggesting that the copy level is probably held constant by autoregulation of polyoma viral T protein synthesis. This vector also amplified expression of the inserted cDNA located on the same plasmid, which results in a higher expression of the gene of interest (Camenisch et al., 1996). Furthermore, this autonomously replicating plasmid can efficiently replicate and maintain a second transfecting *ori*-containing plasmid carrying genes of interest in multiple copies episomally (Gassmann et al., 1995).

- Episomal expression system for gene therapy

Not only feasible in transgene expression experiments, episomal expression systems may also be used in gene therapy for human genetic disorders. Optimal vectors for gene therapy require (a) high-level and stable expression of the transgene, (b) a high transfection efficiency, (c) no integration into the chromosomal DNA to avoid effects on the cell's own DNA and on the vector itself, and (d) no transformation features.

Nonviral vectors have attracted a lot of attention in gene therapy because of the lack of specific immune response, endogenous viral recombination, or oncogenic effects, as can occur with viral gene transfer agents (Conese et al., 2004; Thomas et al., 2003). On the other hand, nonviral vectors have some disadvantages, including low efficiency and the lack of a sustained gene expression. Extrachromosomally self-replicating systems offer multiple advantages in gene therapy, including large packaging capacity and stability without integration. Until now, most progression towards the development of an efficient episomal gene therapy vector has come from Epstein-Barr viral vectors. A non-infectious and non-transforming Epstein-Barr virus-based vector, carrying the entire 185 kb human β-globin gene locus including the control region, has been shown to be maintained as episomes for a period of 3 months in the absence of selection (Black and Vos, 2002). However, the major drawback of such viral-derived episomal expression vectors in the use of gene therapy is the presence of the viral components, while the risk of transforming the transfected cells is still a concern.

4.7.2 Generation of supertransfectable *Pitx3*$^{GFP/+}$ ES cells

Two plasmids, pMGD20neo and pCAGGfpIP (Appendix VIII), are needed to generate the supertransfectable ES cells. Firstly, ES cells are transfected with pMGD20neo and treated with G418 to select cells expressing large T protein (Figure 4-20 A). Then the G418 resistant cells are transfected with pCAGGfpIP, which contains the polyoma *ori* and GFP reporter, and treated with a high concentration of puromycin immediately to select supertransfectable cells (Figure 4-20 B). Among the cells resistant to both G418 and puromycin, those carrying pCAGGfpIP episomally will lose GFP expression gradually when puromycin selection is withdrawn. Then

the GFP negative cells will be collected as supertransfectable ES cells.

$5x10^6$ of *Pitx3*$^{GFP/+}$ (Pt3A) ES cells were electroporated with 20μg supercoiled plasmid pMGD20neo or the mock control at 200V and 960μF in a 0.4 cm cuvette. The cells were then plated $5x10^6$ per 10 cm plate and 200 μg/ml of G418 was added the next day. After 10 days of G418 selection, the mock control plates were clear. One plate from the pMGD20neo group was stained with Leishmann solution and the number of the surviving colonies was counted to establish the transfection efficiency of the parental line. Cells in all other plates were pooled, expanded and stock frozen. These are *Pitx3*$^{GFP/+}$ ES cells carrying the plasmid pMGD20neo, either intrachromosomally or extrachromosomally.

10^6 G418 resistant *Pitx3*$^{GFP/+}$ cells were subsequently plated in one well of a 6-well plate and transfected with a combination of 3μg of supercoiled pCAGGfpIP and 3μl of Lipofectamine 2000 by the lipofection method. After 24 hours the cells were replated at $2x10^5$ or $5x10^4$ cells in 10 cm plates with 200μg/ml of G418 and 2μg/ml of puromycin. After 4 days of double selection, the transiently transfected cells had been cleared and almost all the surviving cells expressed GFP (Figure 4-21 B-B'), which were either supertransfectable or integrated stable transfectants. The colonies which expressed high level of GFP were marked, then puromycin was removed and the cells were cultured in G418-containing medium. Supertransfectable cells, which were carrying the pCAGGfpIP episomally, lost GFP fluorescence gradually. After culturing in the absence of puromycin for 6 days, most cells were GFP negative when examined under a fluorescence microscope. Colonies which were still highly expressing GFP might have the GFP expression vector integrated

intrachromosomally (Figure 4-21 C-C'). At this point the marked colonies which had lost most of the GFP fluorescence (Figure 4-21 D-D') were picked, expanded and stock frozen. The rest of the cells were pooled altogether and sorted for GFP negative cells by FACS. They were then expanded for frozen stocks

A total of 6 lines of supertransfectable *Pitx3*$^{GFP/+}$ ES cells, 5 from colony pick up ($3AT_{C2}$, $3AT_{A2}$, $3AT_{A3}$, $3AT_{A4}$, $3AT_{A5}$) and one by FACS sorting ($3AT_{P}$), were established and expanded. To check the transfection efficiency of these clones, supercoiled plasmid pCAGGfpIP 3μg was transfected into these cells again by lipofection and selected with G418 200 μg/ml plus puromycin 2μg/ml for a total of 10 days. After Leishmann staining, the numbers of colonies per plate were recorded to determine the transfection efficiency (Table 4-3). Compared to the transfection efficiency of their parental lines, these cells had increased transfection efficiency (0.008% vs. 0.02 to 0.49).

4.7.3 Characterisation of supertransfectable *Pitx3*$^{GFP/+}$ ES cells during in vitro differentiation.

Although the ES cells of episomal gene expression system showed high transfection efficiency with *ori*-containing plasmids, these supertransfectable ES cells lost their episomal GFP overexpression plasmids gradually when the high concentration of puromycin were withdrawn. This led to the differentiation experiments for examining whether expression of the gene of interest in the second transfecting *ori*-containing plasmid could be maintained during differentiation into mDA neurons, which takes about 2 weeks. It has been reported that the first plasmid pMGD20neo can be maintained without significant loss for about 50 cell generations in the

absence of selection for G418 resistance (Gassmann et al. 1995). In addition, the episomal maintenance of pMGD20neo is not affected as the cells differentiate in vitro, and in mouse cell lines including mouse L-fibroblasts, renal adenocarcinoma RAG cells and teratocarcinoma F9 cells (Camenisch et al., 1996).

The construct pCAGGfpIP was used as a reporter again and the expression of GFP without antibiotic selection was examined during in vitro differentiation. Briefly, 10^6 cells were transfected with 3μg of supercoiled pCAGGfpIP in one well of a 6-well plate by lipofection. Twenty-four hours after transfection, cells were replated into a 10 cm gelatinised dish with G418 200μg/ml plus puromycin 2μg/ml. Six days after selection, almost all cells that survived expressed GFP. They were pooled into a T25 flask and replated for differentiation on PA6 cells with or without puromycin selection. The interval from the transgene transfection (26/05/05) to the start of the differentiation experiment (04/06/05) was only 9 days, showing their high efficiency. On day 2, 4, 6, 8 of in vitro differentiation, the cells were fixed and counterstained with DAPI. For each experiment 20 colonies were selected randomly and cells counted to determine the percentage of GFP expressing cells. The percentage of cells expressing GFP (GFP^+/DAPI) decreased gradually during in vitro differentiation in the absence of puromycin selection. When differentiated into neural precursor cells on day 8 of in vitro differentiation, only 25.4% of cells maintained GFP expression (Figure 4-22, 4-23). All ES cells kept in puromycin containing medium expressed GFP, but their growth was much slower and rarely differentiated into neural progenitor cells on day 8.

	Transfection efficiency
Parental line	0.008%
Supertransfectable line	
C2	0.49%
A3	0.28%
A4	0.19%
A5	0.08%
A2	0.04%
P	0.02%

Table 4-3 Transfection efficiency of supertransfectable *Pitx3*$^{GFP/+}$ ES cells

pCAGGfpIP was transfected into *Pitx3*$^{GFP/+}$ ES cells carrying the large T protein and selected with G418 and puromycin for 10 days. The numbers of colonies were counted to determine the transfection efficiency. All 6 lines carrying large T demonstrated higher transfection efficiency than their parental line. Line P, which was sorted by FACS, showed lower transfection efficiency than others which were selected by colony pick up.

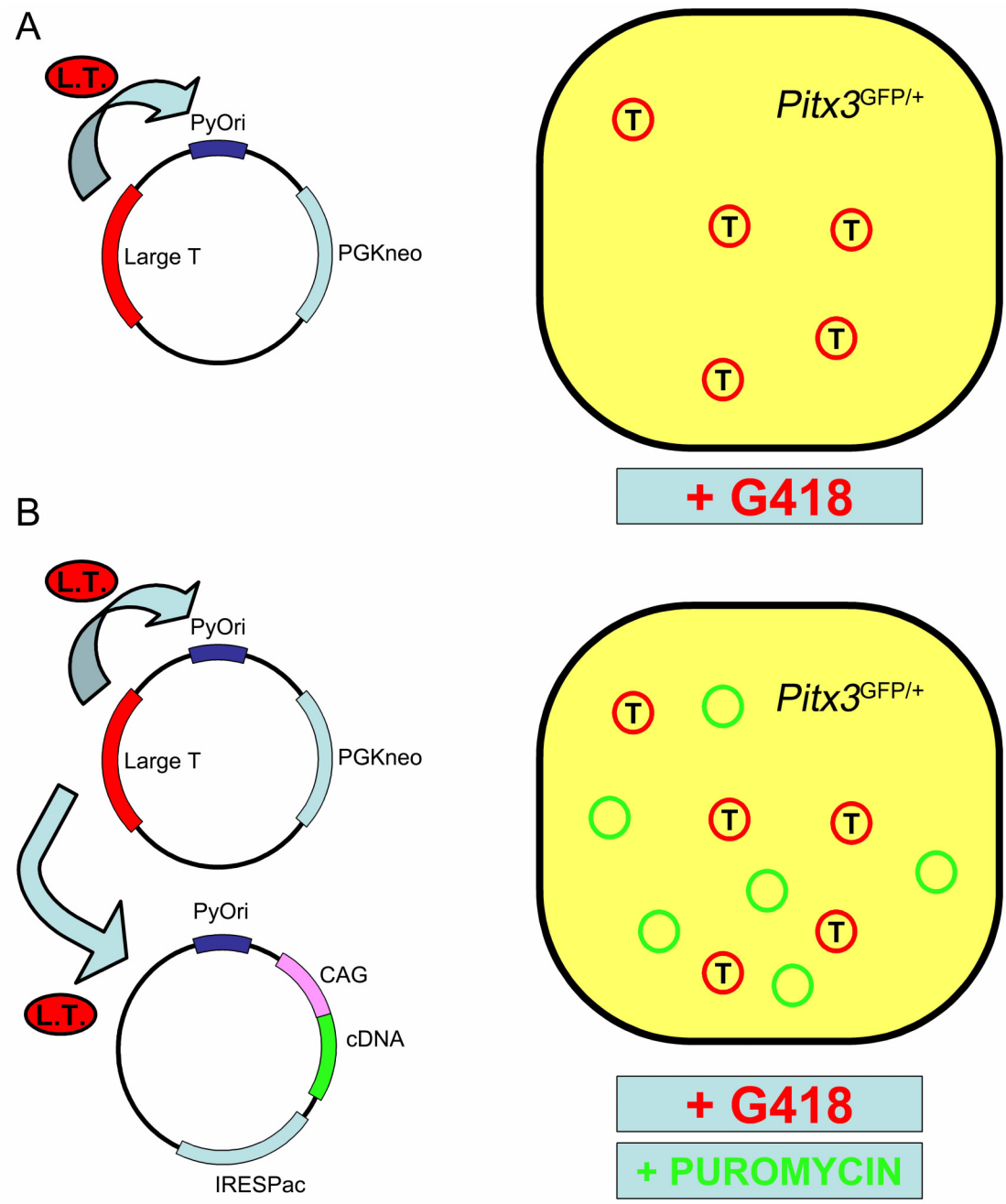

Figure 4-20 Mechanism of the episomal expression system

(A) When introduced into *Pitx3*$^{GFP/+}$ ES cells, the plasmid pMGD20neo will utilise autoregulation to replicate and maintain multiple copies episomally in the presence of G418 selection. (B) The candidate transgene is inserted into a plasmid carrying the polyoma origin of replication. When introduced into cells with a large T protein expressed episomally, the second plasmid will also be replicated and maintained in multiple copies episomally and the transgene expressed at higher levels.

PyOri: polyoma origin of replication, L.T.: large T protein.

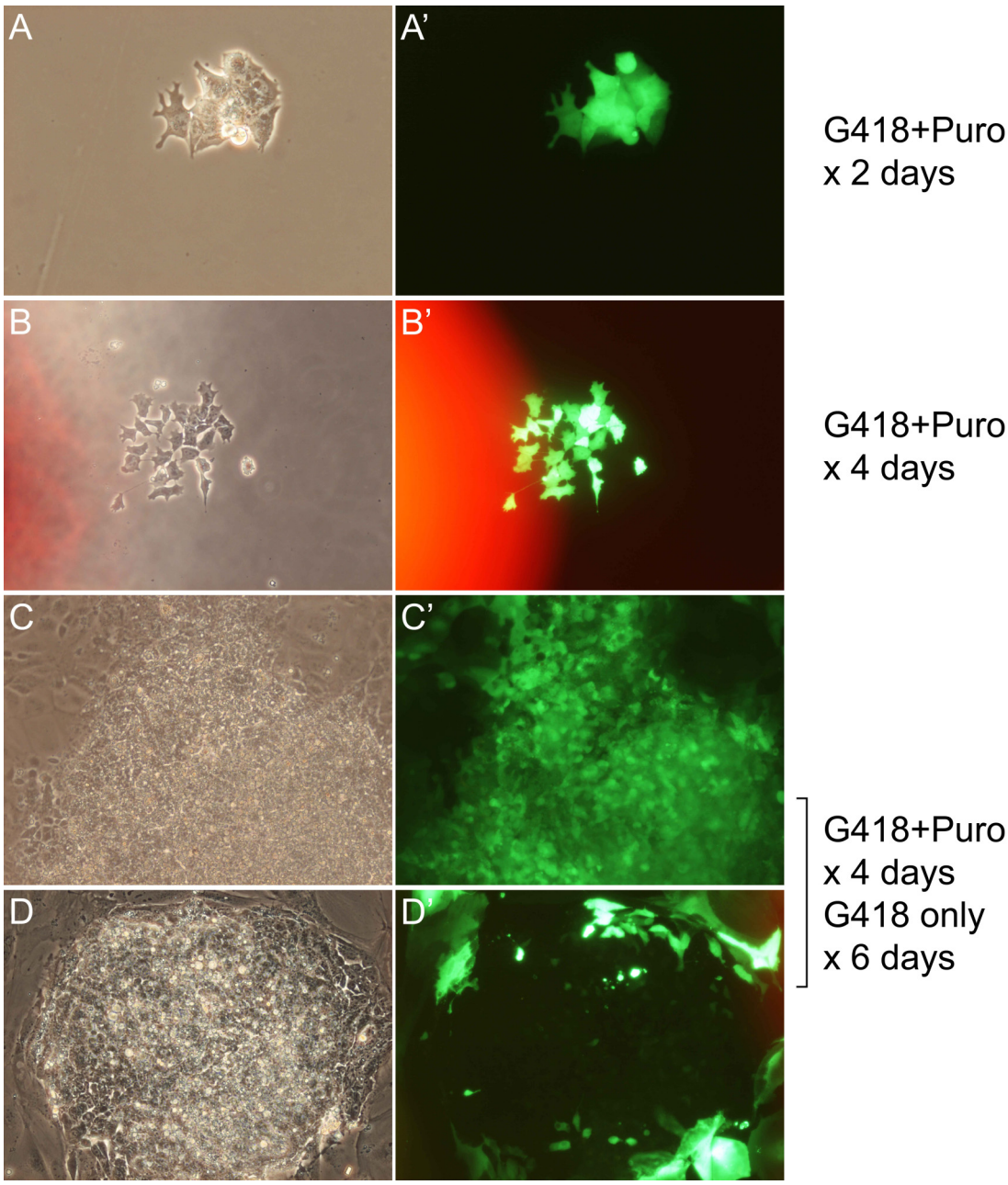

Figure 4-21 Generation of supertransfectable *Pitx3*$^{GFP/+}$ ES cells

Pitx3$^{GFP/+}$ ES cells carrying pMGD20neo were transfected with pPyCAGGfpIP and selected with G418 plus puromycin (A-A'). After 4 days of double selection, colonies which displayed increased GFP expression were marked (B-B'). After culturing in puromycin free medium for 6 days, colonies which still expressed GFP highly might have the GFP overexpression construct intrachromosomally (C-C'). Colonies which lost GFP expression in the absence of puromycin had previously expressed GFP episomally (D-D'). These were the colonies which were picked and expanded.

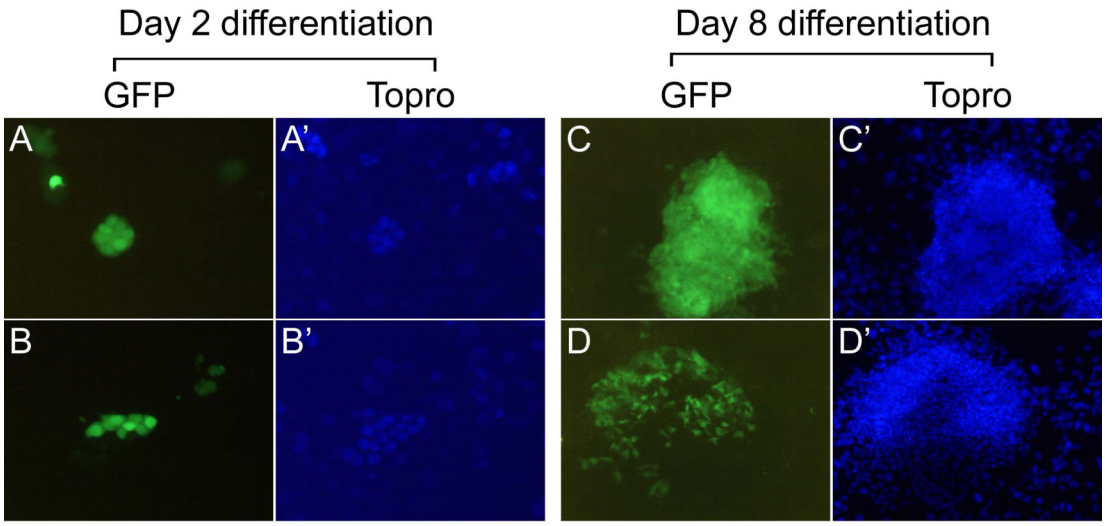

Figure 4-22 Maintenance of transgene expression during in vitro differentiation

The GFP reporter was used to track the maintenance of transgene expression during ES cell differentiation. ES cells transfected with pPyCAGGfpIP were selected in the presence of G418 plus puromycin then in vitro differentiation was performed by use of the PA6 coculture system in puromycin free conditions. Some cells began to lose GFP expression on day 2 of differentiation (B-B'). On day 8 of differentiation, while neural progenitors were derived, some colonies maintained GFP expression (C-C'), but most colonies lost expression of the GFP reporter (D-D'), suggesting that the expression of the second plasmid was lost during differentiation in puromycin free conditions.

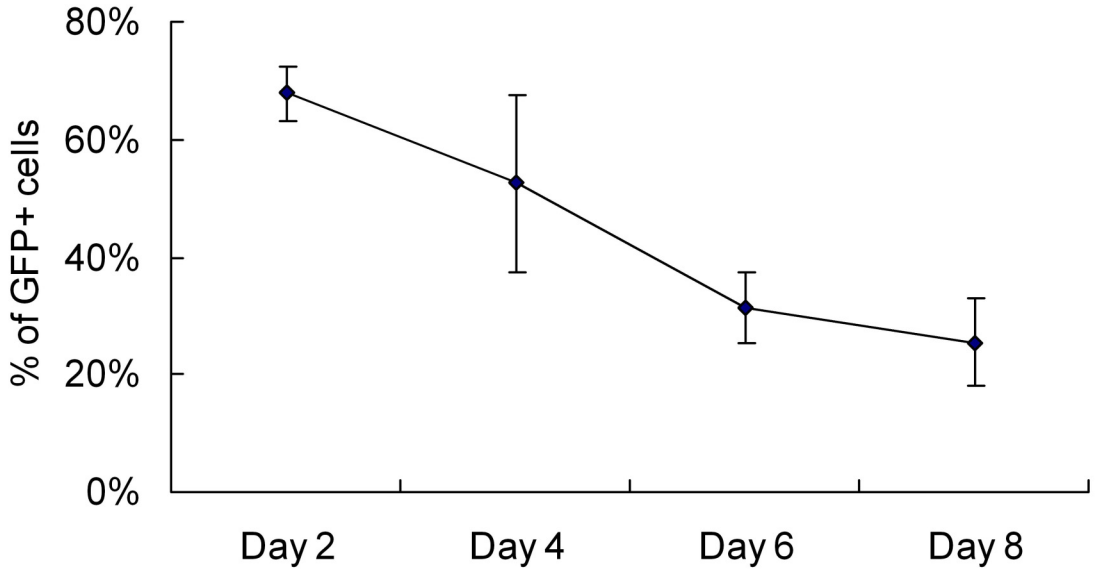

Figure 4-23 Percentage of transgene expression of supertransfectable *Pitx3*$^{GFP/+}$ ES cells during in vitro differentiation.

The number of GFP^{+} cells during differentiation was counted in order to estimate the percentage of cells expressing the transgene. In puromycin free conditions, cells lost the episomally-expressed transgene gradually.

4.8 Discussion

In this chapter, *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ ES cells have been derived from blastocysts. These ES cells can differentiate into *Pitx3*-GFP positive DA neurons with mesencephalic characteristics. The production of *Pitx3*-GFP expressing cells thus can be observed in a cellular system to investigate the molecular and cellular mechanisms involved in mDA development.

4.8.1 Pluripotent mutant ES cell derivation

In the experiments to generate ES cells, I have derived 6 lines of transgenic ES cells from 54 blastocysts and 10 lines of strain 129 ES cells from 70 blastocysts. Among mouse strains, genetic background strongly affects the efficiency of ES cell derivation, therefore the 129 strain, the most permissive for ES cell derivation, has been used as a control group. Brook et al. have shown that the epiblast is the sole source of ES cells in the late blastocysts (Brook and Gardner, 1997). In their experiment, ES cell lines could be obtained from all delayed epiblasts of the 129 strain. They have also suggested that the presence of primitive endoderm impairs the ability of epiblasts to produce ES cells. In the literature, primary ES colonies have been obtained from 25~30% of whole blastocysts in skilled laboratories (Brook and Gardner, 1997; Nichols et al., 1990; Pease et al., 1990). The original derivation protocol (Evans and Kaufman, 1981; Martin, 1981) may be modified to be performed in feeder-free conditions (Nichols et al., 1990; Pease et al., 1990) and even serum-free conditions to produce ES cells without contamination from feeders or serum (Ying et al., 2003a), thus providing proof of principle for suitable sources for cellular replacement therapy. In my derivation experiment, I have used intact delayed blastocysts as the starting material for ES cell production. The ES cell line

yield efficiencies from strain 129 and the mutant group was 16.2% and 13.7%, respectively. The procedure in this experiment was less complicated and the yield acceptable.

After disaggregation from primary ES cell colonies, differentiation was not been completely suppressed for all the lines. In some cases significant numbers of differentiated cells persisted during continuous culture and it was difficult to enrich the undifferentiated cells. Oct3/4 gene regulation has previously been used to overcome the barrier to ES cell production. Since Oct3/4 expression is restricted to undifferentiated ES cells, selection against cells in which the gene is downregulated may enrich the undifferentiated ES cells (McWhir et al., 1996; Mountford et al., 1998). By using this strategy, differentiated cells are continuously removed and germ-line competent ES cells from non-permissive strain can be derived. In my derivation experiment, serum-free ES cell medium has been used to rescue these differentiating lines (Pt2A, Pt2C). Based on the report that BMPs supports ES cell self-renewal in serum-free N2B27 medium (Ying et al., 2003a), differentiated cells did not persist in conditions of serum-deprivation, and the combination of LIF plus BMP4 enhanced the self-renewal of the undifferentiated ES cells. Therefore, undifferentiated ES cell lines Pt2A and Pt2C have been successfully enriched and maintained after being transferred into standard serum-containing medium. However, the long-term stability of these rescued ES cells to be maintained in serum-containing medium and their potential to differentiate in vitro need to be further investigated.

Currently the most practicable way to validate the production of ES cell lines is sustained growth with the retention of a characteristic undifferentiated morphology or antigen expression. It is debatable whether morphology or marker expression provides an adequate basis for identifying genuine ES cells (Gardner and Brook, 1997; Pera and Trounson, 2004). Cell lines from teratocarcinomas also fulfil some ES cell characteristics such as teratoma formation in xenografts in immunodeprived mice, and differentiation into various types of cell in vitro. The identity of ES cells is definitively established by their ability to participate in embryonic development when introduced into early embryos such as morulae or blastocysts. The heterozygous line Pt3A and the homozygous line Pt2B have contributed in high proportions to resulting chimeras (Chapter 3.2), providing further confirmation of their genuine ES characteristics. However, no germ-line competence experiment has been performed.

4.8.2 Midbrain-specific *Pitx3*-GFP positive neurons

Marker analysis including β-tubulin III, TH, En1 and Nurr1, at the single cell level, has demonstrated that the *Pitx3*$^{GFP/+}$ ES cell-derived GFP positive cells show mDA neuron characteristics (Figure 4-4, 4-5). In addition to marker expression, previous work in our laboratory has shown that ES cell-derived *Pitx3*-GFP^{+} mDA neurons are sensitive to MPTP (1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine) treatment, and GDNF treatment can rescue the neurotoxicity, suggesting that they are biologically similar to primary DA neurons in the embryonic and adult midbrain (Zhao et al., 2004).

Many experiments use TH (tyrosine hydroxlase), the rate-limiting enzyme for dopamine synthesis, as a marker for DA neurons. However, in addition to the DA neurons of SN and VTA in midbrain, TH is also expressed in several other cell groups, including DA neurons of hypothalamus and olfactory bulb (Figure 1-3 A), the noradrenergic neurons of the locus coeruleus and lateral tegmental system, and the adrenergic neurons of the brain. In the periphery, TH is expressed in sympathetic ganglia and adrenal chromaffin cells. Therefore, TH alone is not specific enough to identify mDA neurons. My differentiation experiments have revealed that only 13.5% of TH^+ cells co-expressed *Pitx3*-GFP, showing that most ES cell-derived TH^+ cells did not have mesencephalic characteristics. This agreed with previous work in our laboratory that the percentage of GFP^+TH^+ cells in TH^+ population was 13.6%, and this result was generated using *Pitx3*$^{GFP/+}$ ES cells produced by gene targeting of E14TG2a ES cells (Zhao et al., 2004), which were originally derived from the strain 129 (Hooper et al., 1987). Another laboratory has also reported that 21.1% of ES cell-derived TH^+ cells express the mDA marker Pitx3 by the EB method of differentiation (Chung et al., 2005). Recent studies by other members in our laboratory and myself have also revealed that TH^+ neurons derived by the monolayer differentiation method rarely express midbrain markers including *Pitx3*-GFP (Parmar and Li, in preparation). Since different transcriptional regulatory mechanisms may account for regulated expression of the TH gene in different cell populations, a system with an mDA specific reporter such as *Pitx3*-GFP is a more relevant tool with which to study mDA neuronal differentiation.

4.8.3 *Pitx3* null ES cell differentiation

The purpose of deriving homozygous *Pitx3* mutant ES cells lines is to establish a model system to investigate mDA development with loss of Pitx3 protein at a cellular level. In the data obtained from our *Pitx3* null mice, there is a reduction of over 50% in the number of TH expressing cells at E14.5, suggesting *Pitx3* is required for the survival and maintenance of mDA neurons (Maxwell et al., 2005). However, my differentiation experiments of *Pitx3* null ES cells have not shown a significant difference in the number of TH^+ DA neurons generated. This might partly due to our previous finding that a large percentage of ES cell-derived TH^+ neurons are not of midbrain identity (Figure 4-8 B). Indeed, by comparing the percentages of TH^+ population co-expressing *Pitx3*-GFP, I have found a significant reduction of mDA neurons generated by the null cells (Figure 4-8 B), showing an impaired differentiation into mDA neurons.

To further confirm the functional requirement of *Pitx3* for mDA neurons during in vitro differentiation, a replacement study has been performed by stable transfection with a CAG expression vector containing a 1.2-kb full-length *Pitx3* cDNA into *Pitx3* null ES cells. This strategy of rescuing by cDNA expression in double knockout ES cells has been used previously in genetic studies. Such an example is that of Coppolino et al., which show that expression of *calreticulin* cDNA in homozygous mutant ES cells partly rescues integrin-mediated adhesion (Coppolino et al., 1997). Replacing *Pitx3* in the homozygous *Pitx3* null ES cells alleviated the impaired potential to differentiate into *Pitx3*-GFP^+TH^+ neurons in vitro (Figure 4-8 B). These *Pitx3*-GFP positive neurons, which were derived from rescued *Pitx3* null ES cells, also co-expressed an independent mDA marker Nurr1 (Figure 4-9 G-I), suggesting

that they were true mDA neurons, rather than only being auto-regulated by *Pitx3* forced expression. The overexpression experiment has suggested that *Pitx3* might promote the acquisition of midbrain characteristics from DA precursors.

It is worth noting that previous quantitative analysis in our laboratory has revealed that the Pitx3 overexpression *Pitx3*$^{GFP/+}$ ES cell cultures contain around 3 times more *Pitx3*-GFP^{+}TH^{+} than in control cultures. Therefore, the nature of mDA differentiation of the rescued *Pitx3*$^{GFP/GFP}$ ES cells is similar to that of *Pitx3* overexpression in *Pitx3*$^{GFP/+}$ ES cells. This is because the *Pitx3* transgene is overexpressed constitutively by a strong CAG promoter, which does not necessarily mimic the true genetic regulation of *Pitx3* pathway. An alternative rescue strategy to repair the homozygous *Pitx3*$^{GFP/GFP}$ ES cells is to put *Pitx3* cDNA back to its regulatory element by another gene targeting event via homologous recombination, as shown in the *Rag2* study by Rideout et al (Rideout et al., 2002).

The strict correlation in the timing of expression of *Pitx3* and *TH* during development suggests a possible role for the transcription factor Pitx3 as a physiological activator of the *TH* gene. Cazorla et al. have demonstrated the presence of an element within the 100 bp proximal region of the rat *TH* promoter to which Pitx3 protein can bind and through which it can modulate *TH* promoter activity (Cazorla et al., 2000). One high-affinity Pitx3 binding site sufficient for Pitx3 activation of transcription in the mouse *TH* promoter region has also been shown (Lebel et al., 2001). These in vitro promoter studies suggest that Pitx3 protein might contribute to the control of *TH* expression by binding to its promoter. The in vivo mouse studies have shown that, at E12.5, the proportion of TH^{+} cells within the

Pitx3-GFP$^+$ population is significantly reduced in the *Pitx3* knockout midbrain (43.7±5.5%) as compared to their heterozygous littermates (64.8±10.6%, p<= 0.03) (Maxwell et al., 2005), providing the first demonstration that *Pitx3* is a physiological regulator for TH. However, my in vitro differentiation experiments showed no difference of TH expression percentage in the ES cell-derived *Pitx3*-GFP$^+$ neurons between heterozygous and homozygous groups (GFP$^+$TH$^+$/GFP$^+$: 92.8±2.2% vs. 92.2±3.4%, p=0.66). This phenomenon permits several interpretations. Firstly, the regulation of TH by Pitx3 might be very complicated and the question could not be addressed in a simplified in vitro experiment. Similar phenomenon was also observed in En1 overexpression experiments, which will be discussed in the next section. Secondly, the reduction of TH expression in *Pitx3*-GFP$^+$ cells in homozygous *Pitx3* null mice is restricted in SN, rather than in VTA (Maxwell et al., 2005). It has been reported that the level of Pitx3 expression is higher in the VTA than in the SN (Korotkova et al., 2005). Therefore, it is possible that a larger percentage of ES cell-derived GFP$^+$TH$^+$ neurons are of VTA identity. Currently, there is no molecular marker that can clearly distinguish SN from VTA DA neurons. Further investigation is required to clarify this question.

In summary, the *Pitx3* null ES experiments have provided proof of principle that loss of Pitx3 leads to impaired ES cell differentiation into mDA neurons in vitro.

4.8.4 En1 overexpression experiment

The spatial and temporal expression pattern of En1 in mid/hindbrain corresponds to the developmental functions in mDA neurons. Overexpression of En1 has significantly changed the proportion of Pitx3-GFP$^+$ cells in TH$^+$ DA population.

However, the enhancement by En1 overexpression is much lower than by Pitx3 overexpression in heterozygous *Pitx3*-GFP ES cells (Maxwell et al., 2005) or *Pitx3* null ES cells with replacement of Pitx3 (Figure 4-8).

It has been reported that transduction with a lentiviral expression vector carrying En1 during in vitro differentiation significantly induces the expression of the late DA neuron marker DAT, but not the earlier marker TH (Martinat et al., 2006). En1 is associated with survival/maintenance of mDA neurons and the survival effect may depend on signals or trophic support provided by other cells (Simon et al., 2001). My overexpression experiment has suggested that En1 does not enhance mDA development in vitro. Multiple factors may collaborate within a network and En1 alone is not sufficient to regulate mDA development, at least in the ES cell in vitro differentiation system. However, there is still a possibility that En1 regulates components of essential signalling pathways including receptors for required trophic factors. This can be further investigated by exogenous treatment to evaluate whether En1 overexpressing cells are more responsive to neurotrophic factors or more resistant to neurotoxic factors.

4.8.5 Cre-regulated expression system

The Cre-mediated inducible *Pitx3*$^{GFP/+}$ ES cell system developed in our laboratory contains two components: the *Pitx3*$^{GFP/+}$ ES cells which constitutively express CreER^{T2}, and the inducible vector carrying a floxed stop signal (*neoPA*) between the CAG promoter and the transgene. In this chapter the inducible system has been characterised by an inducible vector carrying GFP reporter, which is superior to other reporter assays because it allows sensitive quantification of the number of induced

cells. Before induction, very few cells expressed the GFP reporter. It has also been reported that there is 0.04-0.22% of leakage before 4OHT treatment, detected by GFP reporter as well (Vallier et al., 2001). There are several explanations for this leakage phenomenon. Firstly, the *Gfp* cDNA is occasionally transcribed in spite of the stop signal located in front of it. Therefore it has been suggested to construct more than one transcription termination signal before the transgene to prevent leakage (Mao et al., 2005; Vallier et al., 2001). Secondly, although theoretically $CreER^{T2}$ can only be activated by the synthetic oestrogen analogue 4OHT, the possibility of cross-reacting with certain endogenous ligands cannot be excluded completely. The latter explanation is favoured in my observation since GFP expressing cells have been found to be in groups, suggesting subsequent cell division after unwanted Cre-mediated excision (Figure 4-16 A). Nevertheless, all 4 lines tested showed around 1% leakage rate, suggesting very little influence to this system before induction.

Strikingly, the majority of cells expressed the induced GFP reporter after the treatment of 4OHT, demonstrating a very efficient induction, which includes constitutive expression of $CreER^{T2}$, the activation of $CreER^{T2}$ by the inducer 4OHT, deletion of the floxed stop signal and activation of the transgene by the CAG promoter (Figure 4-14). Further characterisation has also shown high induction efficiency during monolayer neural differentiation. Detailed analysis has revealed that the Cre-mediated system has a precise temporal control of the recombination event during in vitro differentiation although the CAG promoter is less efficient in differentiating cells than in ES cells. However, the whole induced expression machinery is very powerful and a non-detrimental concentration of 4OHT is required.

This inducible system may provide useful research tools for some candidate mDA determinants. For example, data from other members in our laboratory have shown that Klf4 is a candidate mDA regulator but constitutive expression of Klf4 inhibits early ES cell differentiation and complicates an analysis of its function in later development (Li et al., 2005; Takahashi and Yamanaka, 2006). I have constructed an inducible vector (Appendix V), by which a temporally controlled activation of Klf4 may be achieved in ES cell gain-of-function experiments for mDA development.

The conditional transgene approach requires a tight control to avoid unwanted expression and to ensure adequate expression of the candidate gene. To date, two broad categories of conditional transgenesis used in ES cells are site-specific DNA recombination including Cre or Flp recombinase, and transcriptional transactivation including the tetracycline-dependent regulatory system (Gossen and Bujard, 2002; Lewandoski, 2001). In the tetracycline-based system, the tetracycline-controlled transactivator (tTA) is a fusion protein composed of the viral protein VP16 transactivation domain and the E. Coli tetracycline repressor (TetR), which specifically binds both tetracycline and the 19-bp operator sequence (*tetO*) of the *tet* operon in the target transgene and results in its transactivation. In the original system, the tTA cannot bind DNA when the inducer is present ('tet-off'), whereas in a modified version, the 'reverse tTA' (rtTA) binds DNA only when the inducer is present ('tet-on') (Gossen and Bujard, 2002).

In the Cre-based conditional activation system, leaky control does not generate intermediate expression, but will generate a heterogeneous cell population containing both induced and non-induced cells (Figure 4-24 A). By using specific antibodies or

reporters to distinguish the induced and non-induced cells, it is possible to evaluate the cell-autonomous effects of certain candidate regulators on the activated cells and, at the same time, the cell-non-autonomous effects of the regulators to the non-induced cells in the same culture. In the tetracycline-based transcriptional transactivation, or other gene silencing approaches such as RNA interference and dominant-negative methods, leakiness will result in a relatively homogeneous cell population in which quantitative controlled transgene expression is possible (Figure 4-24 B). For example, it has been shown in mouse ES cells that by titrating the expression of transcription factor Oct3/4 within a small margin, three distinct cell fates can be induced (Niwa et al., 2000).

Additionally, the Cre-based approach is irreversible, therefore in this system a pulse of induced Cre activity makes a permanent genomic alteration in a cell and the change is inherited by all the cells' progeny (Guo et al., 2002). This irreversibility prevents the requirement of long-term application of the inducer which can itself have an effect on the phenotype or affect normal cell biology. On the other hand, it is difficult to switch off the activated gene once it has been switched on.

The Cre-mediated inducible *Pitx3*-GFP ES cell system developed in our laboratory is characterised by very low background; that is, the transcription of the transgene is repressed efficiently in the absence of the ligand. The transgene expression in ES cells and their differentiated derivatives can be controlled tightly in response to a non-detrimental dose of the synthetic hormone 4OHT.

4.8.6 The episomal expression system

I have derived supertransfectable *Pitx3*$^{GFP/+}$ ES cells carrying the large T protein. When the supercoiled pMGD20neo construct was introduced into *Pitx3*$^{GFP/+}$ ES cells, not all plasmids were kept episomally. It has been reported that around 15% of G418 resistant transformants carry the plasmid episomally (Camenisch et al., 1996; Gassmann et al., 1995). However, the purpose of this experiment was to generate ES cells with high transfection efficiency, which can be obtained by the GFP reporter test. Therefore I did not check whether the G418 resistant colonies express the large T protein extrachromosomally or intrachromosomally. Theoretically, cells carrying pMGD20neo episomally express the large T protein in multiple copies, thus it is easier for them to express the second transgene in an efficient way. However, it is still possible that some of the lines picked up may carry the large T construct integrated intrachromosomally.

The same observation applied to the GFP expressing cells in the double selection condition. Some of the GFP transgene might be expressed intrachromosomally (Figure 4-21 C-C'), although cells expressing pCAGGfpIP in multiple episomal copies might have a better chance of survival in such a high concentration (2μg/ml) of puromycin. In fact, the original aim of Gassmann et al. to develop the pMGD20neo construct is to determine whether the efficiency of targeting mutations to chromosomal genes in ES cells could be improved by recombination with corresponding homologous DNA segments maintained on episomal vectors (Gassmann et al., 1995). It has been reported that 85% of the double resistant clones contain the second plasmid episomally, suggesting that the pre-existence of large T increases the frequency at which the second transfecting *ori*-containing plasmid can

be maintained as an episome. Cells expressing pCAGGfpIP episomally can be distinguished because they lose GFP expression once the puromycin selection is withdrawn. There are 2 ways to select the GFP negative cells at this point: to pick up colonies which have previously expressed GFP highly, or to sort all GFP negative cells by FACS. In this experiment, the cells sorted by FACS ($3AT_P$) showed lower transfection efficiency than cells selected by colony pick up (0.02% vs. 0.04-0.49%), although still higher than the parental line (0.008%). This is because FACS gives a mixture of GFP negative cells, among which not all have been successfully transfected with pCAGGfpIP. Theoretically, the transfection efficiency can be further enhanced by performing the GFP selection procedure again; that is, to transfect pCAGGfpIP plasmid again and to sort the GFP negative cells after puromycin withdrawal. Another way to get highly transfectable cells is (a) to sort cells highly expressing GFP in the double selection by FACS then (b) to sort GFP negative cells after withdrawal of puromycin. This may select cells which can highly express the transgene efficiently in a quantitative manner.

There are several advantages of episomal expression vectors in transgene expression experiments. Firstly, in stable transgene procedures, the use of episomal vectors results in higher transfection efficiency than the use of a chromosome-integrating plasmid. This feature allows applications that require to transfect large amount of DNA, such as screening of cDNA libraries (Aubert et al., 2002; Chambers et al., 2003). My supertransfectable *Pitx3*$^{GFP/+}$ ES cell study has shown that using 10^6 cells in one well of a 6-well plate for lipofection with a candidate gene, then the number of the transformants will be ready for mDA differentiation experiments within 2 weeks.

Secondly, episomal expression vectors persist in multiple copies in the nucleus, resulting in amplification of the gene of interest. This increases the reliability in gain-of-function expression studies in ES cells that are usually hampered by transcriptional silencing. In normal lipofection experiments for selecting puromycin resistant stable transformants, 0.5~1.0 μg/ml puromycin is added 24~36 hours after replating the transfected cells. However, in the experiment for testing the transfection efficiency of the supertransfectable *Pitx3*$^{GFP/+}$ ES cells, 2 μg/ml puromycin was added immediately after replating. The higher dosage and the shorter interval for adding the selection drug than ordinary usage indicated that the surviving cells were able to express high levels of the resistant gene *pac* in an efficient manner.

Thirdly, the inserted gene of interest is not interrupted or subjected to regulatory constraints, which often occur upon integration into host DNA. The reverse is also true, i.e. the presence of the inserted transgene does not lead to rearrangement or interruption of the cell's own important genomic regions. Integration of foreign DNA into the genome of established cells may have some undesired effects, such as insertional mutagenesis or silencing by *de novo* methylation of the introduced transgene. Using episomal plasmid vectors may achieve high expression levels without affecting the cellular genome (Van Craenenbroeck et al., 2000). This is also an important issue in designing optimal delivery and expression systems in the applications of gene therapy.

Furthermore, because episomal vectors utilise cellular enzymes for replication and repair, they are also powerful tools for studying DNA replication or mutagenesis.

However, there are some drawbacks of the episomal transgene expression system. One disadvantage of the episomal expression system is the interference of viral proteins in host cellular biology. The large T protein of the polyoma virus is known to have a broad spectrum of protein-protein interaction. At the physiological level, the large T protein of the BK virus is able to bind to most of the tumour suppressor protein p53 in the cell (Harris et al., 1996). Simian virus 40 (SV40) large T protein influences cellular gene expression by altering mRNA levels of cellular transcription factors, and also by interacting with the DNA-binding or transcriptional activity of specific transcription factors. It has also been reported that large T proteins block apoptosis induced by growth factor withdrawal in a neural stem cell culture (Harris et al., 1996; Slinskey et al., 1999). Further improvement of these vectors has focused on the elimination of the large T protein. For example, a newly developed SV40 *ori*-based vector, in which the large T antigen is exchanged for the matrix attachment region fragment of the human interferon β-globin, indicates that such a vector remains as an episome in the cell lines of Chinese hamster ovary (CHO) over more than 100 generations without selective pressure (Baiker et al., 2000; Piechaczek et al., 1999).

Another concern is whether the high selective pressure used to maintain the episomal expression will affect some cellular functions. In my differentiation experiments using the GFP reporter, all ES cells kept in puromycin selection on puromycin resistant PA6 cells expressed transgene GFP, but the time course of differentiation was delayed and rare neural differentiation was observed at day 8 of culture. This suggests that puromycin treatment might affect normal ES cell differentiation in vitro.

The main drawback of the episomal expression system is a gradual loss of transgene on the second *ori*-containing vectors during differentiation in the absence of selection. Only around 25% of transgene expression is maintained at day 8 of differentiation when neural progenitor cells are derived. Therefore, the episomal expression system is more suitable for testing potential early determinants during differentiation. Successful examples include the identification of Nanog that maintains ES cell self-renewal (Chambers et al., 2003), and the Wnt antagonist Sfrp2 (secreted frizzled-related protein-2) that affects early neural differentiation from ES cells (Aubert et al., 2002). My own experiment showed that, the transgene expression levels at day 2 and day 4 of differentiation are still 67% and 52%, respectively (Figure 4-23), which might be applicable for certain candidate analysis.

It is feasible to deal with the problem of the maintenance of transgene expression by engineering a detectable marker, i.e. HA-tag or Flag-tag, or other fluorescence protein, to track the transgene expression. It has been reported that 15% of the second *ori*-containing plasmid is integrated intrachromosomally (Camenisch et al., 1996). In my GFP reporter experiment, around 7% of colonies highly expressed GFP after 2 weeks of differentiation in the absence of puromycin. Most of these colonies might have the GFP expression vector intrachromosomally. By constructing a detectable marker for reporting the transgene expression, the cells or colonies maintaining the transgene expression can be distinguished, and the effects of the candidate gene on the production of *Pitx3*-GFP expressing cells can be determined. Another alternative is to construct candidate genes into the first plasmid carrying the large T protein. High expression of the transgene inserted in the same plasmid carrying the large T protein has also been reported in the absence of selection

(Camenisch et al., 1996). This is also the main principle in the design of viral-derived self-replicating vectors for gene therapy for human genetic disease (Conese et al., 2004; Van Craenenbroeck et al., 2000)

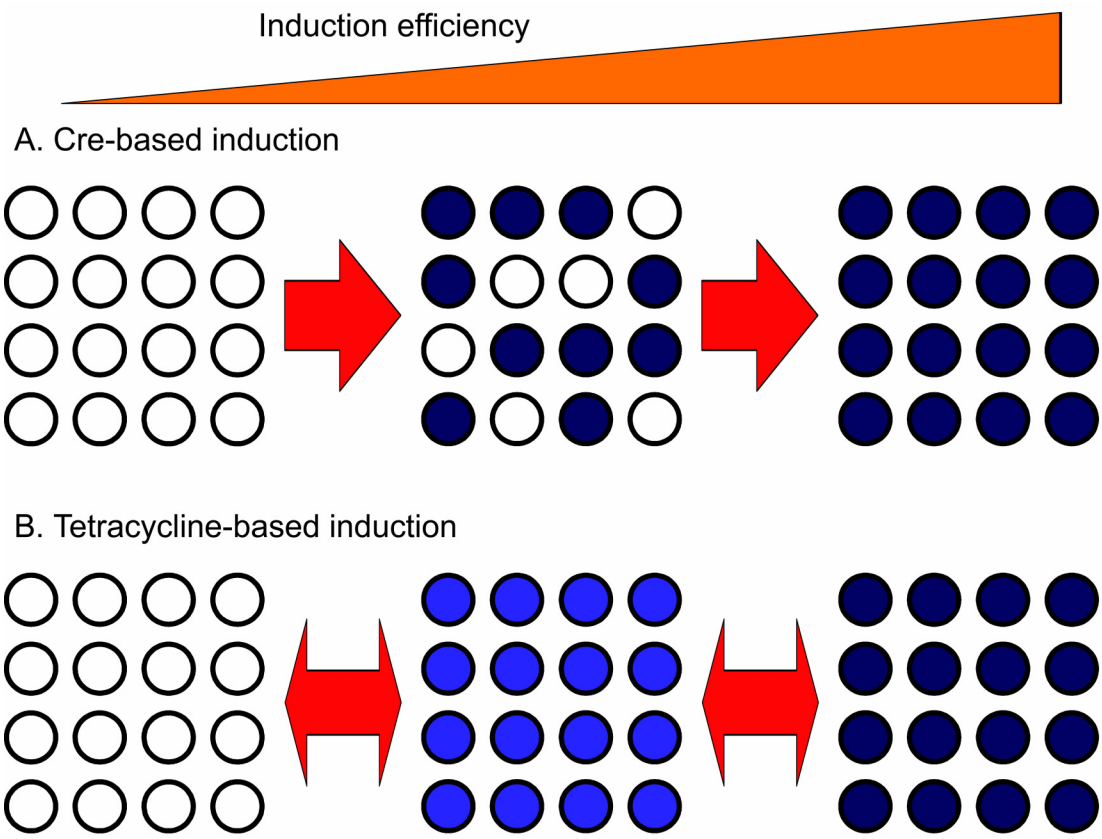

Figure 4-24 Comparison of the Cre-based induction strategy to the tetracycline-based system

(A) Cre-base site-specific recombination strategy may generate a heterogeneous cell population containing both induced and non-induced cells in which the cell-autonomous and non-cell-autonomous effects may be evaluated. This approach is irreversible thus preventing long term application of the inducer which may itself have an effect on the cell biology.

(B) The tetracycline-based transcriptional transactivation may result in a relatively homogeneous cell population in which intermediate transgene expression is possible. This system is reversible, so it is possible to switch off the transgene which has been activated.

Circles represent cells expressing normal (dark blue), intermediate (light blue) or no (white) transgene products.

Chapter 5

General discussion

5.1 Distinct functional roles of Pitx3 in the developing midbrain and lens

Impaired development of both mDA neurons (Maxwell et al., 2005) and lens in *Pitx3* null mice has been demonstrated. Interestingly, the transcription factor Pitx3 shows distinct functional roles between the developing midbrain and the developing lens (Figure 5-1).

In this thesis I have shown that the expression of Pitx3 in the developing lens is restricted to the lens epithelial layer and the bow region where cell proliferation proceeds. The *Pitx3* null lens showed expanded expression of the cell cycle inhibitors $p27^{KIP1}$ and $p57^{KIP2}$, suggesting that Pitx3 might prevent cell cycle arrest. On the other hand, Pitx3 is expressed in post-mitotic mDA neurons. The expression of *Pitx3*-GFP and Sox1, which is a marker of proliferative neural progenitors (Pevny et al., 1998), has been found to be mutually exclusive in the E12 midbrain (Zhao et al., 2004). Additionally, *Pitx3*-GFP positive mDA neurons do not express Ki67, a proliferative marker which labels cells in G1, G2 and M phase (Maxwell et al., 2005). These data suggest that *Pitx3*-GFP expressing cells in the midbrain have withdrawn from cell cycle. The reduced number of GFP^+TH^+ cells in $Pitx3^{GFP/GFP}$ mice suggests that Pitx3 is required for generation and maintenance of the post-mitotic mDA neurons, while in developing lens Pitx3 is required for the maintenance of the proliferative lens epithelial cells.

Furthermore, the reduced number of midbrain TH expressing cells in the *Pitx3*-GFP positive population in $Pitx3^{GFP/GFP}$ mice provides biological evidence that Pitx3 is also involved in the regulation of TH, a key enzyme for the synthesis of dopamine, in agreement with the in vitro promoter studies that Pitx3 is able to bind to a response

element in the TH promoter and confer a transcriptional effect (Cazorla et al., 2000; Lebel et al., 2001). This is in contrast to its function in developing lens where Pitx3 might be a repressor for the activation of fibre cell-specific crystallins.

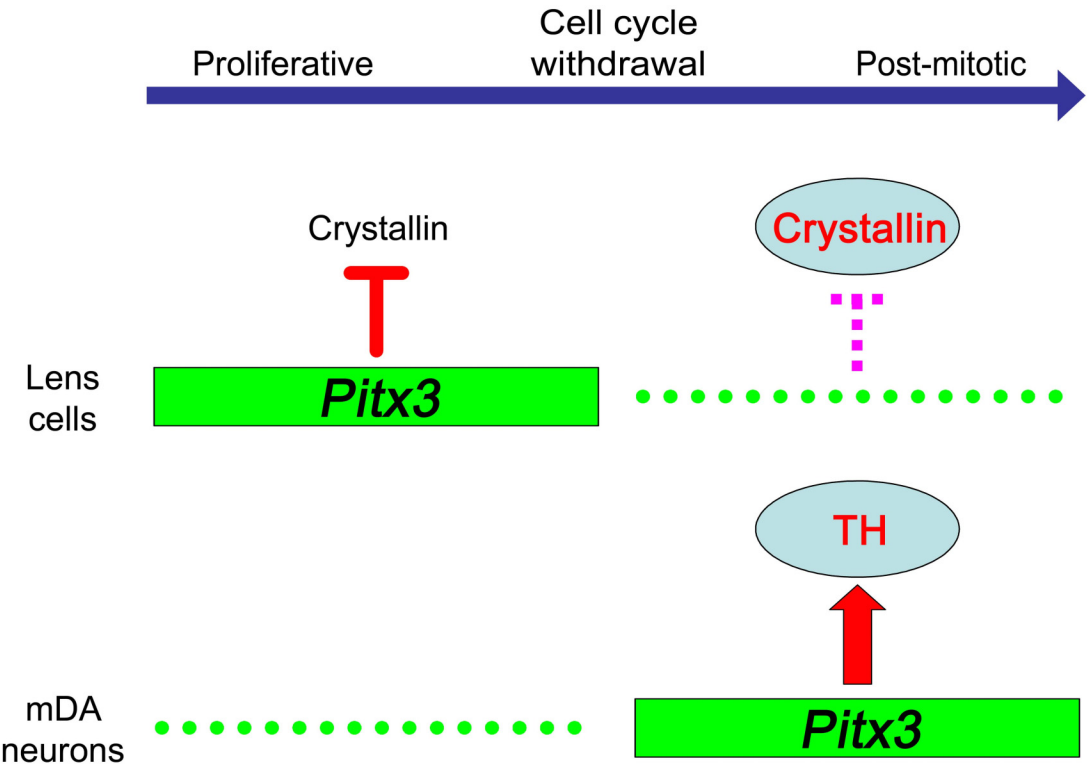

Figure 5-1 Distinct functions of Pitx3 in developing midbrain and lens

Pitx3 is expressed and required in proliferative lens epithelial cells before cell cycle withdrawal, and in post-mitotic mDA neurons of midbrain. Functionally, it may also be a repressor of lens fibre cell-specific crystallin expression. On the other hand, Pitx3 regulates the expression of TH in mDA neurons.

5.2 Further application of the *Pitx3*-GFP reporter ES cells

The homozygous *Pitx3*$^{GFP/GFP}$ ES cells can differentiate into GFP positive cells with neuronal morphology and expression of TH and Nurr1. It is feasible to check other expression profiling to see if *Pitx3* has regulatory roles for other transcription factors or signalling pathways. Additionally, the homozygous *Pitx3*$^{GFP/GFP}$ ES cells have shown impaired differentiation potential into mDA neurons, and can be rescued by Pitx3 replacement (Chapter 4.4). Therefore, the Pitx3 candidate downstream targets can also be tested in this cellular system by forced expression in *Pitx3* null ES cells to examine whether the impaired differentiation into mDA neurons is rescued. Thus *Pitx3* null ES cells provide a platform to screen candidate targets functionally before further complicated animal studies.

One interesting candidate pathway is the regulation of apoptosis in mDA neurons. Previously in our laboratory it has been shown that loss of mDA neurons in *Pitx3* null mice is probably due to apoptotic cell death during development (Maxwell et al., 2005). The TUNEL assay has shown that around double the number of *Pitx3*-GFP$^+$ cells in the *Pitx3* null SN are undergoing apoptosis compared to the *Pitx3* heterozygous SN and VTA or the *Pitx3* null VTA. The primary culture of E14.5 midbrain has also revealed that around double the number of *Pitx3*-GFP$^+$ cells in the *Pitx3* null culture are undergoing apoptosis as compared to the *Pitx3* heterozygous culture. However, further molecular mechanisms remain elusive. It will be interesting to put caspase-3 inhibitors into these ES cell culture to see if the anti-apoptotic treatment rescues the phenotype. Furthermore, it is also applicable to introduce an expression vector carrying Bcl-x_L, an anti-apoptotic protein of the Bcl-2 family which is involved in cell survival of SN mDA neurons (Savitt et al., 2005;

Shim et al., 2004).

Lineage-specific reporters provide an opportunity to use purified, molecularly defined cell populations from developing midbrain or ES cell-derivatives for testing the functional reconstitution in Parkinsonian brain, and for uncovering the molecular identity of the desired 'donor' cells for developing cell replacement therapy in treating PD. For example, GFP reporter driven by *Ngn2*, which is expressed in DA neuron precursors in the developing midbrain, has been used to identify transplantable cells from mouse developing ventral midbrain. It has been shown that *Ngn2*-GFP$^+$ cells from mouse midbrain give rise to a higher ratio of TH$^+$ cells in PD animal transplantation studies, compared to cells expressing GFP under the control of an earlier marker *Nestin*, demonstrating an enrichment in the progenitor pool in terms of the number of survival DA neurons (Thompson et al., 2006). Additionally, glia cells in donor ventral midbrain or ES cell-derivatives might also help DA neuron development and survival in transplantation animal experiments (Hall et al., 2003). FACS-sorted *Ngn2*-GFP expressing cells from developing ventral midbrain, which have committed into neuronal fate, did not show a significant increase in DA neuron numbers in grafts compared with the unsorted grafts. It has been suggested by the authors that the enrichment of DA progenitors might be countered by the poor survival of DA neurons in grafts lacking ventral midbrain glia cells (Thompson et al., 2006).

Further transplantation experiments in animal models are needed to establish the type and developmental stage of cells that is required for functional repair in Parkinsonian brain, with regard to tumourigenicity and developmental plasticity. *Pitx3*-GFP

reporter ES cells may represent a valuable tool for advancing studies in this area. *Pitx3* expressing cells are post-mitotic mDA neurons (Maxwell et al., 2005; Zhao et al., 2004), thus having the lowest tumourigenicity when transplanting into host midbrain. On the other hand, they might have the lowest developmental plasticity, which is also a significant issue for functional recovery of cell replacement therapy in neurodegenerative disease. However, *Pitx3*-GFP reporter ES cell-derivatives, from different differentiation methods or of different stages, may be used in transplantation experiments to track the survival of 'right' type DA neurons in Parkinsonian brain, which has been shown as an important factor for functional repair of PD animals (Hudson et al., 1994).

Instead of the ES cell-based research system, other cells in the *Pitx3*-GFP reporter mice may also be used in cellular and molecular research of mDA development. Primary culture of ventral midbrain cells at E11, when *Pitx3*-GFP is not expressed, can be used for testing exogenous factors or signals which can promote development of *Pitx3*-GFP^{+} neurons. Furthermore, cells from other lineages in *Pitx3*-GFP reporter mice may also be used for testing their potential to trans-differentiate into *Pitx3*-GFP positive mDA neurons, which is of special interest in the search for alternative sources to ES cells for cell replacement therapy.

5.3 Concluding remarks

The experiments in this thesis provide knowledge in developing lineage-specific reporter ES cells. First of all I have derived heterozygous and homozygous *Pitx3*-GFP reporter ES cells from mutant blastocysts. The *Pitx3*$^{GFP/+}$ and *Pitx3*$^{GFP/GFP}$ ES cells have been used in chimeric animal experiments and demonstrated a cell-autonomous requirement of Pitx3 in developing lens epithelium. These

experiments have led to further molecular characterisation of *Pitx3* null lens which reveals premature activation of fibre cell differentiation programme of lens epithelial cells, suggesting that Pitx3 is a key transcription factor for the maintenance of lens epithelium, partly via the regulation of the transcription factor Foxe3. Clearly there are many factors involved in lens epithelial cell maintenance and fibre cell differentiation that are yet to be uncovered. Future studies also need to address the relationships and interactions between Pitx3 and other transcription factors or signalling pathways involved in lens development. Acquiring this knowledge may lead to a better understanding of the role that transcription factors play in mouse and human ocular development.

On the other hand, in vitro differentiation experiments have shown that the ES-derived *Pitx3*-GFP positive cells are DA neurons of mesencephalic identity. I have generated supertransfectable *Pitx3*$^{GFP/+}$ ES cells to offer a rapid and efficient way for introducing and expressing a transgene episomally. The Cre-mediated inducible system of *Pitx3*-GFP reporter ES cells has also been developed in our laboratory and I have shown that they have high induction efficiency thus allowing transgene activation in a temporally controlled manner. The homozygous *Pitx3*$^{GFP/GFP}$ ES cells showed impaired potential to differentiate into mDA neurons and may potentially be used for evaluating candidate Pitx3 downstream targets by gain-of-function test. The purpose of developing a *Pitx3*-GFP reporter ES cell system is to identify key determinants governing mDA fate and to investigate mechanisms of regulatory genes in mDA neuronal differentiation. Additionally, understanding how to generate appropriate cell types robustly and under defined conditions from ES cells will be essential for functional applications including the development of cell

replacement therapy.

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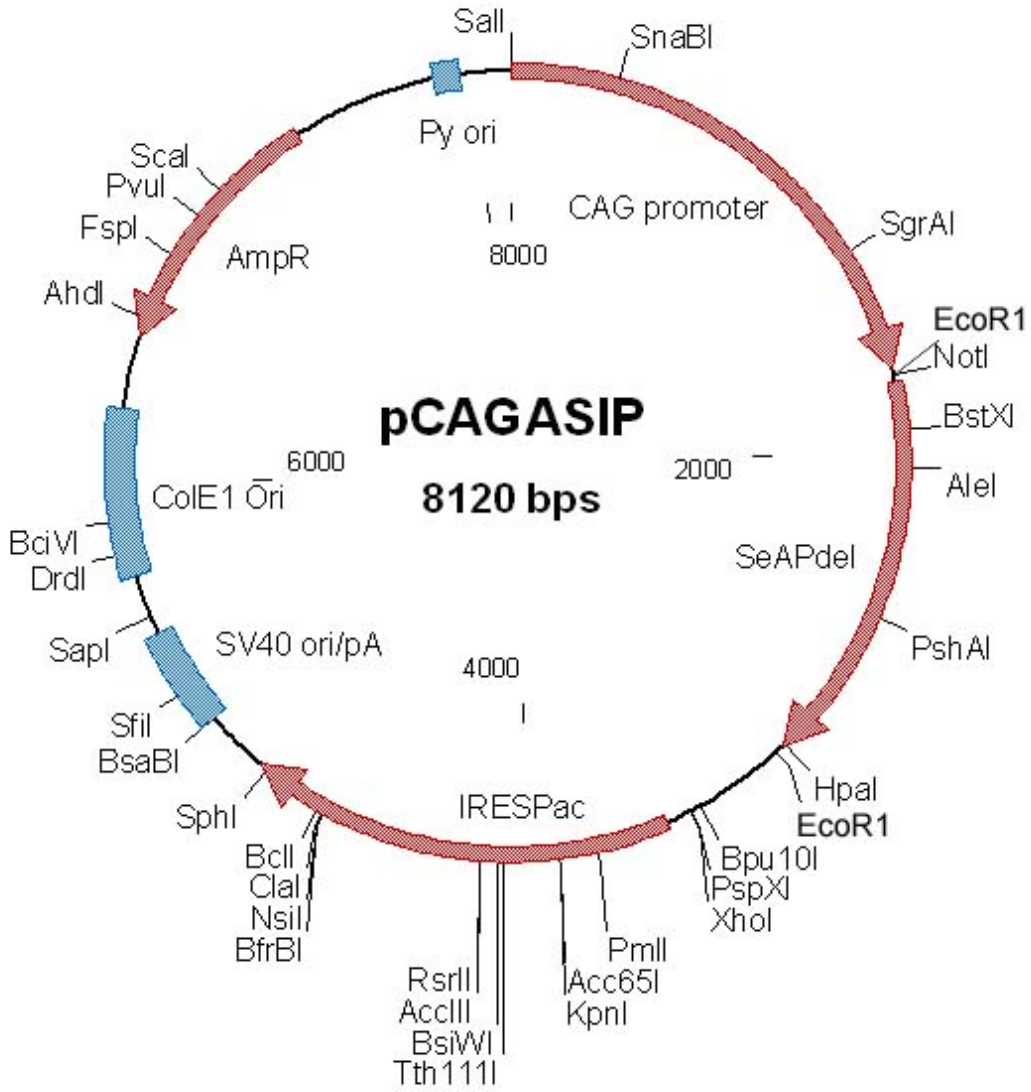
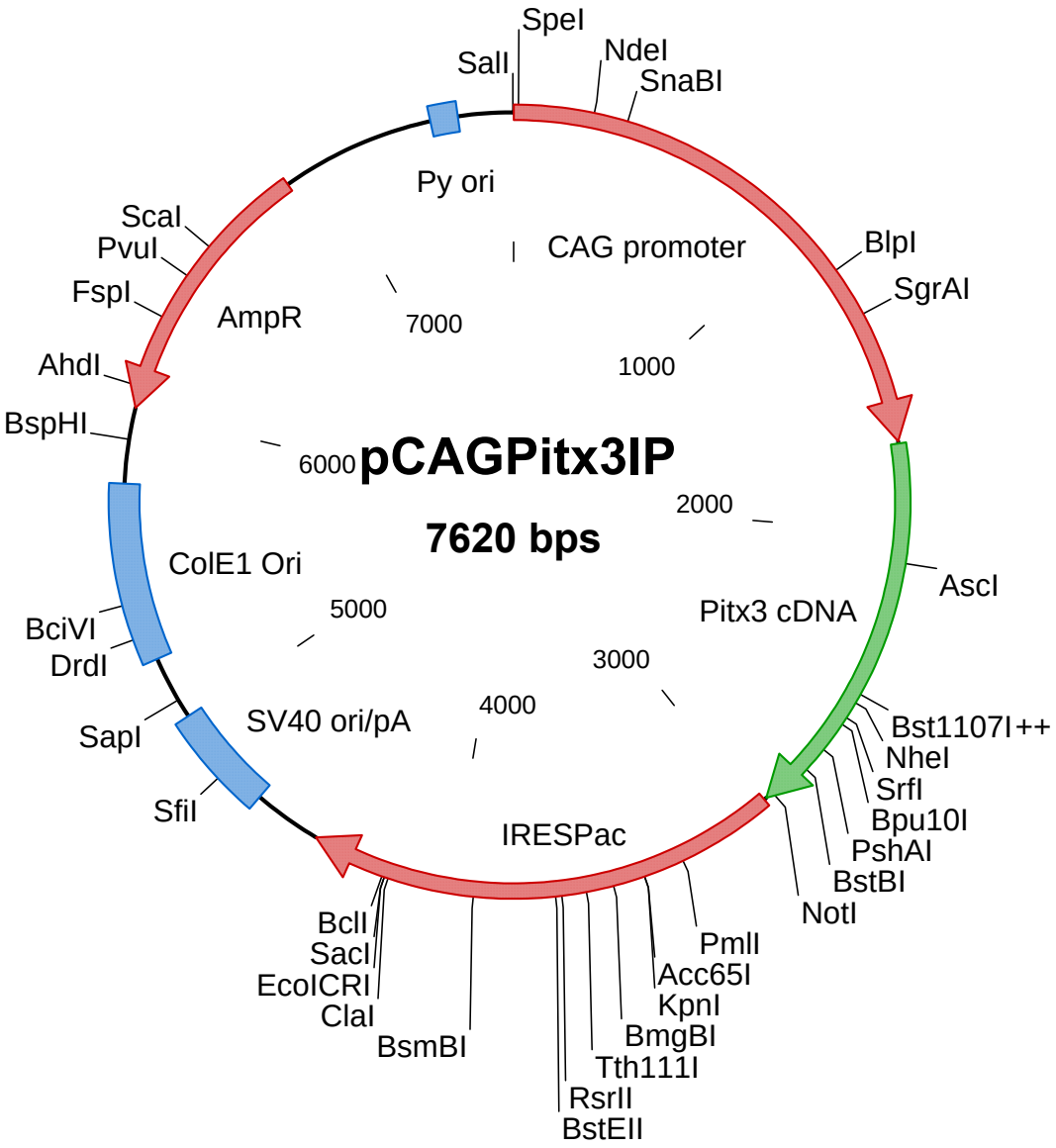
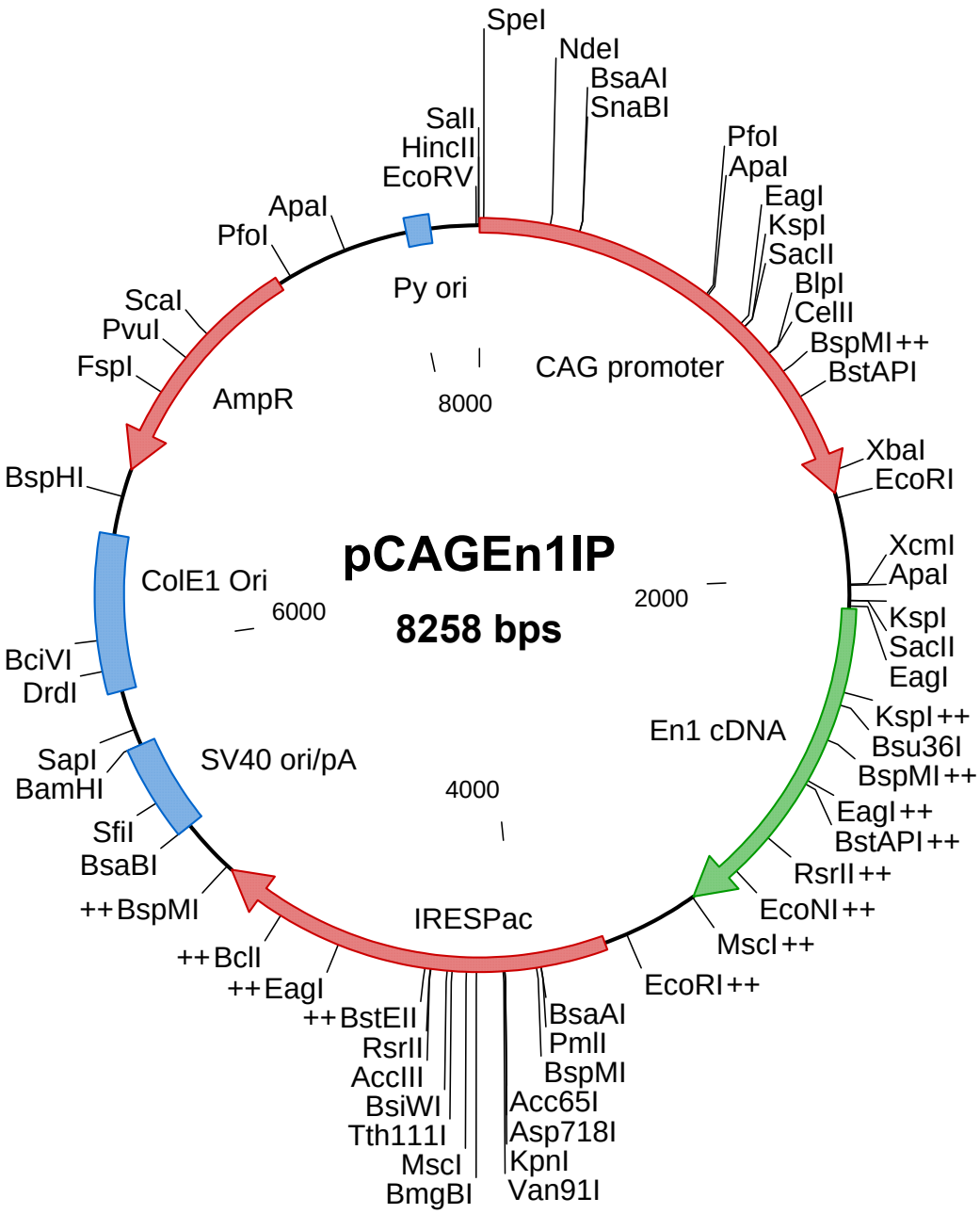
Appendix I pCAGASIP plasmid map



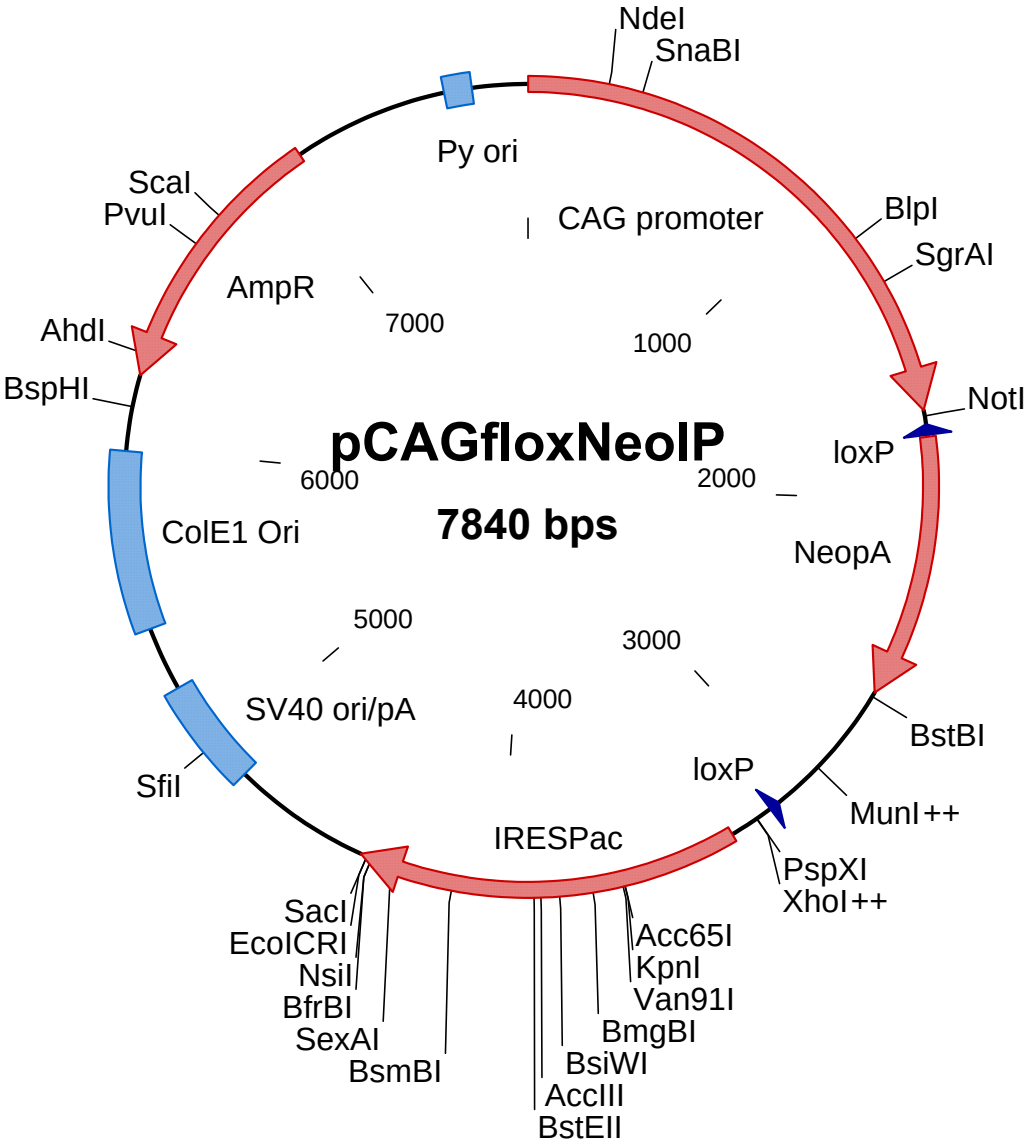
Appendix II pCAGPitx3IP plasmid map



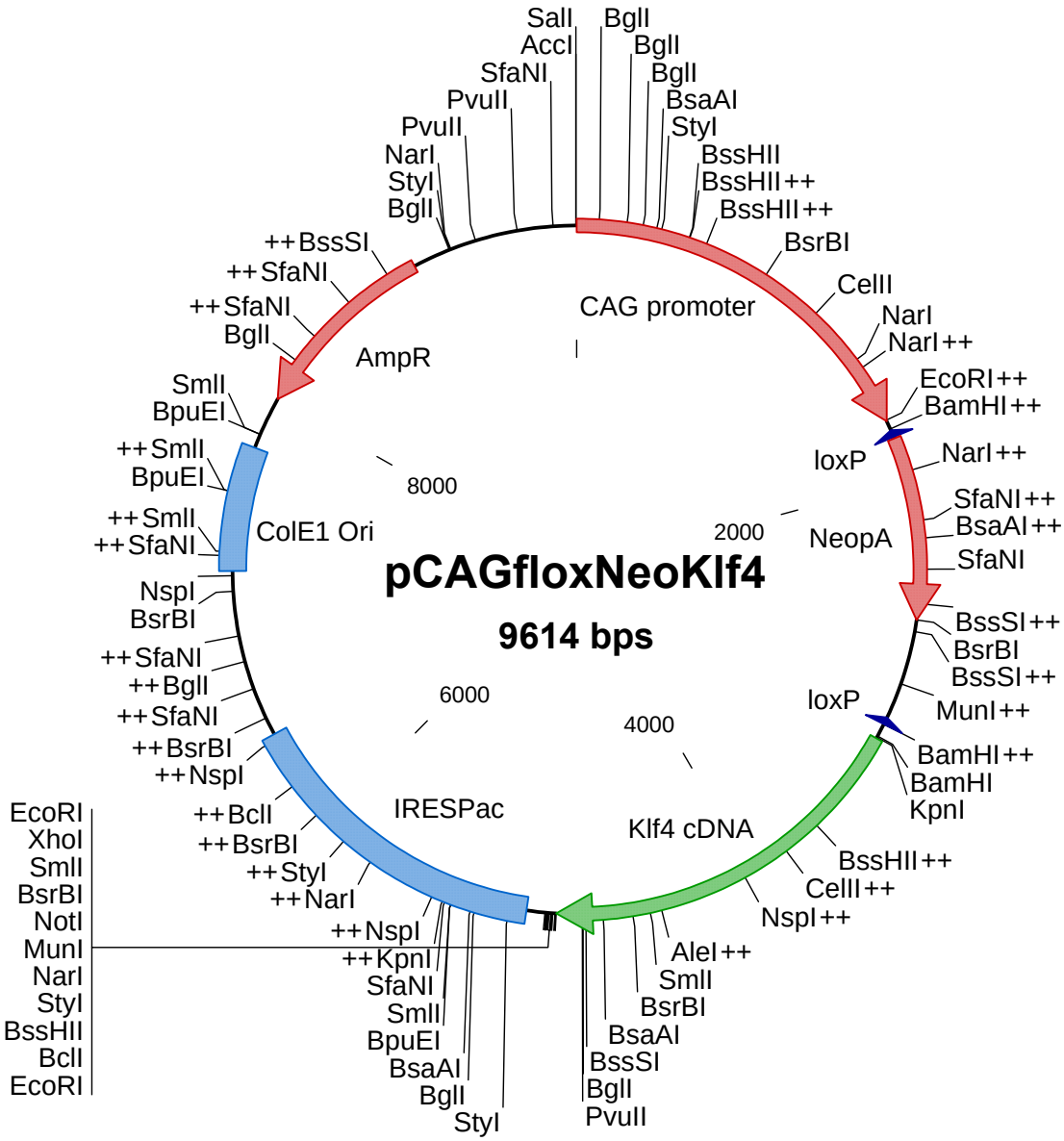
Appendix III pCAGEn1IP plasmid map



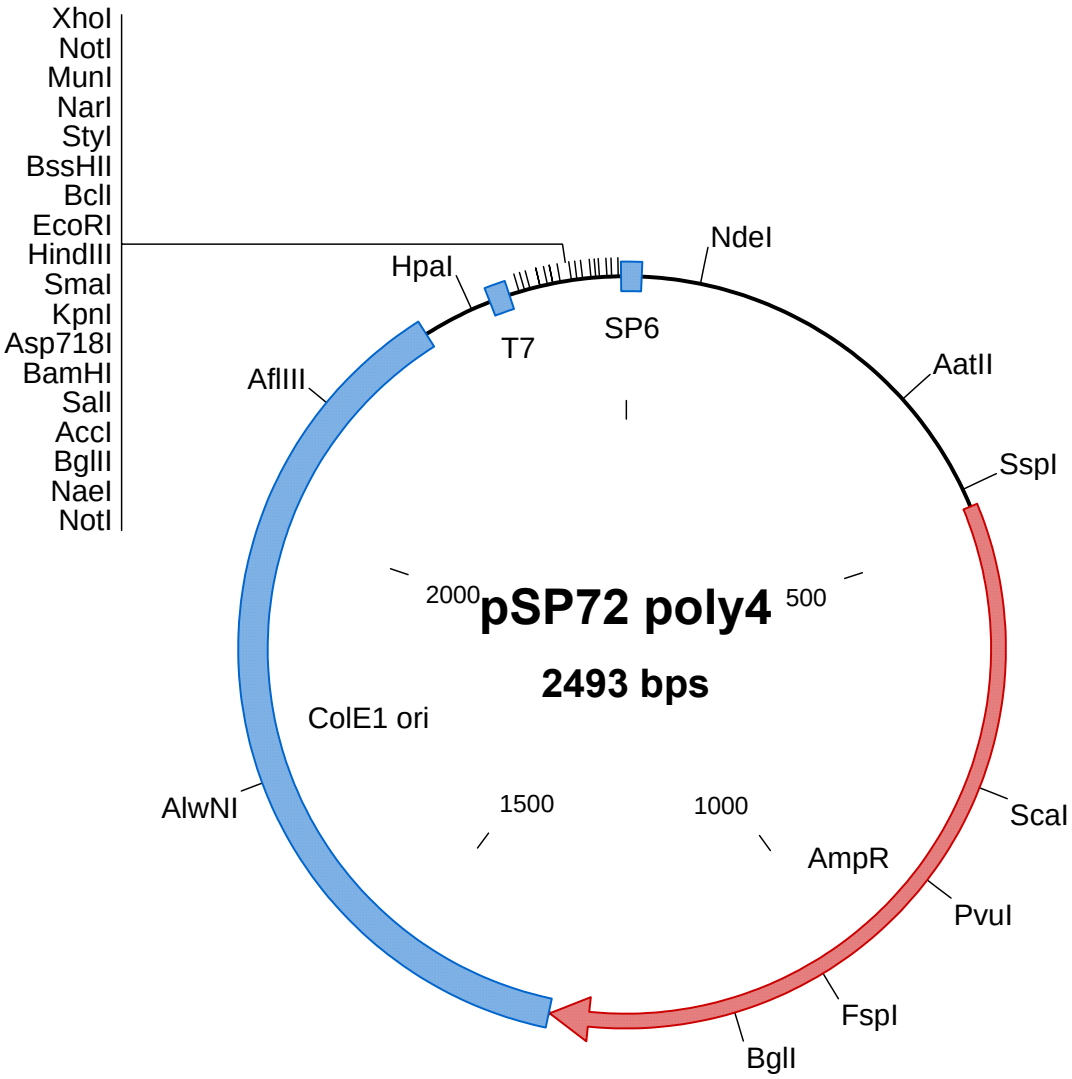
Appendix IV pCAGfloxNeoIP plasmid map



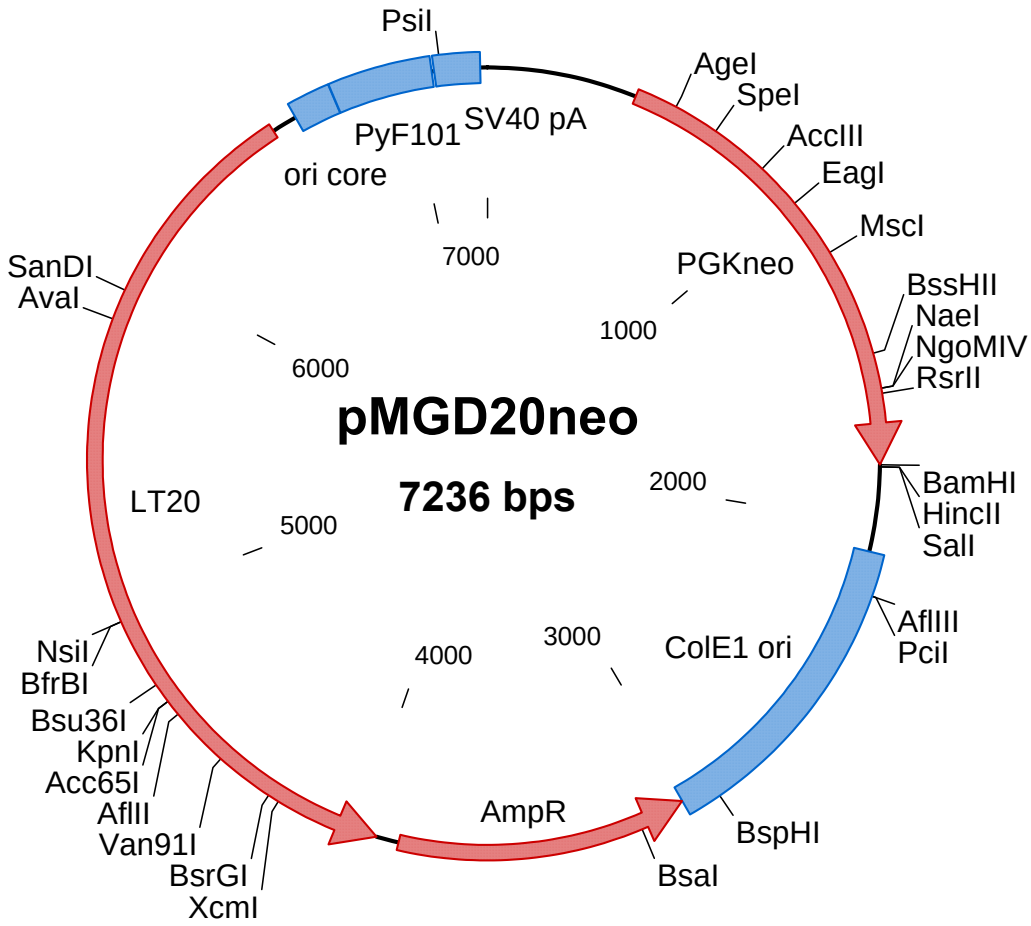
Appendix V pCAGfloxNeoKlf4 plasmid map



Appendix VI pSP72poly4 plasmid map



Appendix VII pMGD20neo plasmid map



Appendix VIII pCAGGfpIP plasmid map